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METABOLISM OF PARASITIC HELMINTHS[1]

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IN HIS BOOK, *An Introduction to Comparative Biochemistry*, Baldwin (13) has commented on the disproportionality between our knowledge concerning the biochemistry of vertebrates and bacteria on one hand and that of invertebrate metazoa on the other. This author has pointed out that biochemistry is "in danger of growing without properly differentiating, of developing members adult in size while possessing only the most primitive supporting structures and correlating apparatus," and that "only by ensuring the proportionate growth of all its members can the full humanitarian value of biochemistry be realized."

As with other invertebrate metazoa, information about the metabolism of parasitic helminths is very limited. On the other hand, the distribution of parasitic worms is extremely wide. At least 800 million human beings are infested with helminths and in many of them multiple infestations occur; Stoll (176) has estimated that the total number of helminthiases exceeds two billion. The metabolism of parasitic worms, as a group, is of particular interest also, because of the great differences in the chemical composition of the habitats of various species. In view of these marked environmental differences which include also differences in the oxygen tension, it is not surprising that the metabolism of these organisms varies from one species to another to a much greater extent than does that of the vertebrates.

Several subjects discussed in this article have been reviewed previously by MacCoy (125), Von Brand (199, 210), Von Brand and Jahn (204), Lapage (113), Smyth (170) and Hobson (93).

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PROBLEM OF OXIDATIVE METABOLISM

Since many parasitic worms live in the intestinal tract where the oxygen tension is low it has often been assumed that the metabolic activities of these organisms in their physiological habitat are primarily, if not exclusively, anaerobic. Indeed, it has been postulated by some investigators (227, 161, 111, 233) that oxidative reactions are not essential for their survival. Others (59, 163, 164, 127, 46, 114, 187) have categorically rejected such a possibility. The presently available evidence does not permit this controversial question to be answered by a generalization.

Parasitic helminths cannot be considered as obligatory anaerobic organisms, since they will take up oxygen if it is available to them (171, 52, 5, 2, 81, 88, 89, 195, 110, 26, 23, 25, 203, 205, 211, 150) and since they will survive in environments whose oxygen tension is high. However, many of these organisms have access only to a small amount of oxygen, because their habitats, such as the intestinal tract or the bile ducts, contain very little of this gas (180, 193, 195, 182, 22, 121). An exception may be found in the case of the intestinal parasites of the pig, because the oxygen tension in the gastrointestinal tract of this animal frequently is considerably higher than in that of other mammals (193, 195). Since the rate of respiration of several intestinal helminths varies directly with the oxygen tension of the medium (81, 87, 109), respiration of these organisms must be extremely small at the low oxygen tensions usually prevailing in the intestinal tract. Furthermore, large intestinal helminths such as *Ascaris* and certain tapeworms can survive under anaerobic conditions for relatively long periods of time (227, 90, 233, 31, 19, 183, 171, 52, 177, 237, 31). Only when frequent convulsions of *Ascaris* are induced by electrical shocks do these parasites die more rapidly in an oxygen-free atmosphere than in air (163). Therefore, aerobic metabolism appears to supply the additional energy required by heightened muscular activity. However, under physiological conditions such activity must occur but rarely, if ever, since, according to radiological evidence, the motility of *Ascaris* in the human intestinal tract is very low (115). Consequently, it appears unlikely that the large helminths which inhabit the intestinal tract and the bile ducts derive a major portion of their energy requirements from aerobic reactions. On the other hand, it cannot be stated that the small amount of respiratory metabolism presumably occurring at the low oxygen tensions of their habitat is of no physiological importance. More precise information about the significance of their respiratory metabolism could be obtained by the development of nutritionally adequate media for culturing these parasitic worms aseptically *in vitro*. This would make it possible to investigate their response to various oxygen tensions and to anaerobiosis over prolonged periods of time.

Special conditions under which oxygen is supplied to intestinal helminths exist in the host-parasite relationships of two types of nematodes: *1*) Wells (235) has shown that the hookworm, *Ancylostoma caninum*, obtains oxygen from oxyhemoglobin by sucking the blood of the host; the amount of blood withdrawn by these nematodes is larger than is commonly appreciated, and at least in part, may account for the anemia observed in this parasitic infestation (64). *2*) If the intestinal parasite is of sufficiently small size diffusion of oxygen into the organism may occur even at low oxygen tensions. This appears to be the case with the nematodes,

Ostertagia circumcincta and *Trichostrongylus spiralis*, which live in the digestive stomach (abomasum) of sheep (45, 46). As pointed out by Von Brand (199), these organisms are about 30,000 times smaller than *Ascaris* and therefore, the ratio of surface area to volume of such nematodes favors a high rate of respiration, even at low oxygen tensions. The available evidence indicates that respiratory metabolism is essential for these two types of intestinal parasites which have access to large amounts of oxygen: Davey (45, 46) has demonstrated that certain small nematodes, *Ostertagia circumcincta* and *Trichostrongylus spiralis*, are extremely sensitive to lack of oxygen and that they survive for periods five to fifteen times as long in an atmosphere of air as under anaerobic conditions. In addition, according to the findings of Von Brand and Otto (198), the carbohydrate metabolism of hookworms resembles that of non parasitic worms which depend on aerobic metabolism to a much greater degree than *Ascaris*. Recent observations of Rogers (152) indicate that aerobic mechanisms also play an important role in the metabolism of other small intestinal nematodes (*Nippostrongylus muris*, *Nematodirus spp.* and *Hoemonchus contortus*).

The habitats of tissue helminths usually have a higher oxygen tension than those of intestinal worms. However, a relatively high oxygen tension of their environment does not necessarily indicate that such organisms are greatly dependent on respiratory metabolism. This is illustrated by the differences in the behavior of two tissue helminths, the filarial nematode, *Litomosoides carinii*, and the trematode, *Schistosoma mansoni*. The former lives in the pleural cavity, the latter in the blood plasma of the mesenteric and portal veins. The oxygen tension of the pleural cavity varies between 12 and 25 mm. Hg (74, 224, 44, 75, 178), that of the portal vein between 49 and 66 mm. Hg (17, 51, 92). Through the use of the cyanine dyes (21, 234, 239, 138), it has been possible to investigate to what degree respiratory metabolism is essential for these two parasites. The cyanines in extremely low concentrations inhibit the oxygen uptake of the filariae (234, 26, 138) and of the schistosomes (25). This effect of the cyanines is not restricted to conditions *in vitro*; if the dyes are administered to the mammalian hosts, the respiration of the parasites is markedly inhibited (24, 26). Reduction of their respiratory metabolism results in the death of the filariae (234, 26), but the schistosomes survive (24, 25) and continue to produce eggs (139) under these conditions despite the fact that the oxygen tension of their habitat is higher than that of the filariae. Furthermore, anaerobiosis or inhibition of respiration produced by the cyanine dyes results in a compensatory increase in the rate of glycolysis of the filariae (26), i.e., a much larger proportion of the carbohydrate utilized by the organism is converted to lactic acid. With *S. mansoni*, the production of lactic acid from glucose is the same in the presence or absence of oxygen, or of cyanine dyes (25). The possibility cannot be excluded that *S. mansoni* may require a small proportion of its normal oxidative metabolism. Although this organism survives when 80 per cent of its respiration is inhibited by a cyanine dye, death might occur if respiration were to be inhibited completely. In this eventuality, the difference, in their respective dependence on oxidative metabolism, between *L. carinii* and *S. mansoni* would be a quantitative rather than a qualitative one, and the oxygen requirement of *S. mansoni* would be comparable to that of another tissue parasite, the larval nematode of *Eustrongylides ignotus*. Von Brand and Simpson (208) found that this organism survives at relatively low oxygen tensions in nutritionally ade-

quate media for many months, but dies rapidly under strictly anaerobic conditions. Another tissue nematode, the larval form of *Trichinella spiralis*, appears to require oxidative metabolism for the preservation of its motility, but not for its survival (171).

Harnisch (80, 83, 85, 87, 82), Friedheim and Baer (67), and Von Brand (211) have demonstrated a direct proportionality between the oxygen tension of the environment and the rate of respiration of parasitic worms. This is observed also when minced tissue, instead of the whole organism, is used (80, 82), indicating that a more rapid diffusion of oxygen at higher tensions does not account for this phenomenon. On the other hand, the oxygen uptake of tissues from predominantly aerobic organisms, such as vertebrates, insects, crustaceans and many free-living worms, is much less dependent on the oxygen tension, their respiration remaining constant above a certain minimum level of the oxygen tension (84, 86, 80, 82, 122, 96, 38a). On the basis of these observations, Harnisch (83, 85, 87, 82) has postulated that any organism whose tissue respiration, under optimal conditions of diffusion, is dependent to a large degree on the oxygen tension, has a predominantly anaerobic type of metabolism in its physiological habitat. Should this hypothesis prove to be correct, a simple experimental procedure would be available to determine whether the metabolism of a given organism is primarily aerobic or not. However, according to observations of Van Grembergen (187, 189) respiration of the tissue pulp of *Moniezia benedini* and of *Fasciola hepatica*, two helminths possessing a predominantly anaerobic metabolism, is independent of the oxygen tension over a wide range. Furthermore, the respiration of the filarial worm *L. carinii*, which has a predominantly aerobic type of metabolism, is at least as dependent on the oxygen tension as the respiration of *S. mansoni*, whose metabolism is primarily anaerobic (27). Consequently, the relationship between the oxygen uptake of a parasitic helminth and the oxygen tension cannot be considered as a reliable criterion for the relative importance to the parasite of anaerobic and aerobic metabolism (212).

The evidence discussed in the foregoing paragraphs supports the conclusion of Von Brand (199, 210) that the dependence on respiratory metabolism varies greatly from one parasitic helminth to another and that it must be investigated for each individual species. With some of them, the requirement for oxidative reactions is high and short periods of anaerobiosis or partial inhibition of their oxygen uptake results in their death. Others can survive when the oxygen tension of their habitat is extremely low, or when their respiration is almost completely inhibited. For some of the latter, respiratory metabolism may not be essential at all and may be merely a rudimentary function carried over from a previous stage of the life cycle of the parasite, or from a different, but phylogenetically related species.

CARBOHYDRATE METABOLISM

With the exception of *Ancylostoma caninum* (198), of microfilariae of *Wuchereria Bancrofti* (20) and of adult *Trichinella spiralis* (132), parasitic helminths in general are characterized by a high glycogen content. In many respects the glycogen isolated from *Ascaris lumbricoides* is similar to that from mammalian tissues (62, 226, 9). The same appears to be true for the polysaccharide present in *Monieza expansa* (131), *Fasciola hepatica* (131), *Cysticerus fasciolaris* (153), *Macracanthorhynchus hirudinaceum* (197, 202) and *Litomosoides carinii* (26). Utilization of glycogen is an important

mechanism by which parasitic worms obtain energy. Absence of carbohydrate from their environment results in a pronounced decrease in the glycogen content of the parasites. This was discovered in *Ascaris lumbricoides* by Weinland (227) and subsequently has been confirmed for this organism (195, 111, 213, 196, 161) as well as for all other parasitic helminths whose glycogen metabolism has been investigated (223, 183, 200, 26, 194, 142, 143, 144, 124). Incubation of extracts of *Ascaridia galli* and of *Nematodirus spp.* results in a decrease in endogenous glycogen (151). Utilization of endogenous glycogen during starvation of parasitic worms has been demonstrated not only *in vitro* but also within the host. Reid (142–144) has observed that the glycogen contents of two intestinal helminths of the fowl, the cestode *Rallietina cesticillus* and the nematode *Ascaridia galli*, are reduced to about 10 per cent of their original values after starving the host for 20 to 24 hours. On the other hand, the starvation or the feeding of large amounts of glucose to dogs infested with *Ancylostoma caninum* had no effect on the glycogen stores of these hookworms (198). In this particular case the parasites obtain their food by blood-sucking (235) and are thus independent of nutrients supplied by the intestinal contents.

Less quantitative data are available on the metabolism of simple sugars by parasitic worms. Weinland (227) has reported that the glucose content of *Ascaris lumbricoides* decreases during starvation. However, Foster (65) and Von Brand (195) did not detect any significant amounts of reducing sugars in *Ascaris*. According to Von Brand (195, 210), the high glucose values preceding starvation reported by Weinland (227) were artefacts produced by hydrolysis of glycogen during the determination of free reducing sugar. Glucose, fructose, and, to a lesser extent, galactose and maltose, increase the period of survival of *Fasciola hepatica* in a buffered salt solution, but lactose and sucrose were ineffective (172). Survival *in vitro* of the larval *Eustrongylides* is prolonged by glucose, but is not affected by fructose, mannose or maltose (206). When added to the medium or when injected, glucose, and to a lesser degree, fructose or galactose, have a glycogen-sparing effect on *Ascaris lumbricoides* (229). An increase in the glycogen content of *Ascaris lumbricoides* by the injection of glucose was not observed consistently (229). It is possible that glycogen synthesis from glucose and other substrates could be demonstrated more readily if the glycogen stores of this organism were depleted by starvation. A glycogen-sparing effect of glucose was observed also in *Moniezia expansa* (194) and the larval *Eustrongylides* (208). Addition of glucose to the medium resulted in a slight increase in the glycogen content of *Moniezia expansa* (222). This effect was not observed with fructose, maltose, galactose, arabinose, glycine or mucin (222). Markov (124) has reported a very pronounced decrease in the glycogen content of the plercocercoids of the cestodes *Diphyllobothrium latum*, *Ligula intestinalis*, *Eubothrium rugosum* and *Triaenophorus nodulosus* during incubation in Ringer-Locke solution. Addition of glucose to this medium produced a slight increase in the glycogen content of the parasites. A very marked rise in the glycogen stores of the filarial worm *Litomodoides carinii* was observed when this organism was incubated aerobically in a glucose-containing medium (26). Polysaccharide synthesis by extracts of *Ascaridia galli* in the presence of adenylic acid and glucose-1-phosphate has been demonstrated by Rogers (151). Such an observation suggests that glycogen synthesis in this organism involves enzymatic mechanisms similar to those discovered by Cori and his coworkers (39–42,

69) in mammalian tissues. The tissue parasites, *L. carinii* and *Schistosoma mansoni*, have a very high rate of glucose utilization. The Schistosomes can remove, from their surrounding medium, in one hour an amount of glucose equivalent to almost one fifth of their dry weight (23, 25). The rate of glucose utilization by *Litomosoides carinii* is about half as great as that of *Schistosoma mansoni* (26). These two helminths appear to have the ability to oxidize hexoses since the addition of glucose or fructose produces an increase in their respiration (26, 27). Similarly, the oxygen uptake of *Diphyllobothrium latum* was greatly increased by glucose (67). On the other hand, glucose did not increase the respiration of *Moniezia benedini* (187) or of larval *Trichinella spiralis* (171). Glucose utilization by *Schistosoma mansoni* was

TABLE 1. PRODUCTS OF THE CARBOHYDRATE METABOLISM OF PARASITIC HELMINTHS

SPECIES	PRODUCTS
Ascaris lumbroides	n-valeric acid
	acetic acid
	lactic acid
	formic acid
	butyric acid (?)
	propionic acid (?)
	aldehydes (?)
	ethyl alcohol (?)
Parascaris equorum	valeric acid
	lactic acid
Moniezia expansa	higher fatty acids
	lactic acid
	succinic acid
Fasciola hepatica	higher fatty acids
	volatile fatty acids
Larval *Trichinella spiralis*	CO_2
Schistosoma mansoni	(D,L)-lactic acid
Litomosoides carinii	L (+)-lactic acid
	acetic acid
Dracunculus insignis	lactic acid

found to be independent of the glucose concentration in the medium. On the other hand, the rate of fructose utilization by this organism was directly proportional to the concentration of this sugar (27).

The observations summarized above suggest that several hexoses, notably glucose, can be converted to glycogen by parasitic helminths and that they can replace this polysaccharide as a fuel. Under physiological conditions the habitats of parasitic worms contain an adequate supply of glucose for these reactions.

Lower volatile and higher fatty acids are the main products of the anaerobic carbohydrate metabolism of intestinal helminths. Weinland (227, 231) isolated the acids produced by *Ascaris lumbricoides* as their calcium salts and concluded that this parasite converts glycogen predominantly to valeric acid and to a smaller extent to caproic acid. This was confirmed by Flury (62). According to this author, *Ascaris lumbricoides* also produces butyric acid, propionic and small amounts of formic acid. In addition, several colorimetric tests suggested the presence of aldehydes in media

in which these worms had been incubated (62). Von Brand (195) found that this helminth produces small, but measurable amounts of lactic acid. Waechter (214) isolated, from media in which *Ascaris lumbricoides* had been incubated, an acid whose copper salt was obtained in crystalline form. The shape of these crystals was identical with that of copper-n-valerate but showed no resemblance to the copper salt of iso-valeric acid. The preparation and identification of the phenyl-phenyl-acyl derivative of n-valeric acid from similar media by Oesterlin (130) has established that this acid is a major product of the carbohydrate metabolism of *Ascaris lumbricoides*. It is interesting to note that valeric acid is also a product of the metabolism of the free-living worm, *Lumbricus terrestris* (117). In addition to n-valeric acid Oesterlin (130) demonstrated the production of acetic acid by *Ascaris*. While formation of butyric acid could not be established conclusively, strong evidence for the production of formic acid was obtained by this author (130). He found no evidence that *Ascaris* produces iso-valeric acid. Circumstantial evidence that ethyl alcohol is a product of the carbohydrate metabolism of *Ascaris* has been reported (62, 105).

According to Toryu (184), the major product of the carbohydrate metabolism of *Parascaris equorum* is valeric acid and only a small fraction of the acids formed by this organism can be accounted for by lactic acid. These observations confirmed the earlier work of Fischer (59). Von Brand (194) found several differences between the metabolic products of *Ascaris lumbricoides* and those of *Moniezia expansa*. The latter organism produces no volatile acids, but large quantities of higher fatty acids, some of which appear to be monohydroxy acids (194). Furthermore, Von Brand (194) observed that *Moniezia* forms succinic acid and greater amounts of lactic acid than *Ascaris*. Lactic acid production accounted for 16 per cent of the glycogen utilized by *Moniezia expansa*. Weinland and Von Brand (223) and Stephenson (173) have found that higher fatty acids are also the major products of the carbohydrate metabolism of *Fasciola hepatica*. This trematode, unlike *Moniezia*, produces small amounts of volatile fatty acids.

In addition to the above-mentioned products, it has been reported frequently that parasitic helminths form carbon dioxide from carbohydrate. CO_2 production may be due either to the actual production of this gas or to the formation of an acid or acids which liberate CO_2 from bicarbonate present in the medium or preformed in the helminth. Furthermore, CO_2 liberated by either of these mechanisms may be partially bound and retained by the proteins or other buffers of the organism (219). Unless corrections are made for these two sources of error, a quantitative evaluation of CO_2 production is not possible.

Slater (163, 164) has stated that fatty acids present in solutions in which parasitic helminths had been kept were not produced by these organisms, but by bacterial contamination. The evidence available does not support such an hypothesis: *1*) Although *Ascaris lumbricoides* and *Moniezia expansa* live in the same habitat, and, presumably, carry the same bacterial contaminants, different metabolic products are formed by these two parasites. *2*) Higher fatty acids not only are present in the medium in which *Fasciola hepatica* has been kept, but they also have been demonstrated by histochemical methods within the excretory system of this organism (192, 223), and, in *Ascaris*, the same lower fatty acids which accumulate in the medium can be isolated from the body of the worm itself (62, 157). *3*) In many experiments

the worms were kept in a salt solution containing no glucose or other substrate. It appears quite improbable that significant fatty acid production by bacteria could occur in such non-nutrient media. Even when glycogen or glucose had been added to such media, no increase in valeric acid content occurred after removal of *Ascaris* (231). *4)* Slater (163) quotes experiments of Fischer (59) which indicate that, in contrast to intact *Parascaris equorum*, the only acid formed by extracts from this animal is lactic acid. However, in these experiments, the total acidity of the extracts was determined by boiling them before titration. Thus, volatile acids originally present in the extracts could have been driven off by such a procedure. Furthermore, Fischer could detect no significant lactic acid formation when his extracts were incubated for 15 hours at 37° C. Only after this period did a rapid increase in lactic acid occur. Such an observation suggests that the production of lactic acid was due to bacteria (119) and not to the activity of the tissue extract itself, since the enzymatic activity of the latter presumably decreases rather than increases with time. *5)* Weinland (228) has shown that volatile fatty acids were formed by extracts of *Ascaris lumbricoides* to which antiseptics had been added. Sterility tests revealed the absence of bacteria in these extracts (228). *6)* It has been pointed out above that the rate of acid production, associated with the growth of contaminating bacteria, should increase with time. As shown by Toryu (184) the formation of volatile acids during incubation of *Parascaris equorum* remains constant over prolonged periods of time.

A type of metabolism, as yet unexplored, which differs from that of other parasitic helminths, is found in *Trichinella spiralis*. Stannard, McCoy and Latchford (171) have observed that these organisms produce a considerable amount of carbon dioxide, but no organic acids whatsoever. While the adult and larval forms of *Trichinella* are alike in their lack of production of acids, the adult trichina contains, in contrast to the larvae, little or no glycogen (132). Investigation of the carbohydrate metabolism of larval and adult trichinellae should prove of great interest and might reveal some hitherto unknown metabolic reactions.

Until recently it appeared that, in contrast to tissues of vertebrates and to many bacteria, the major portion of the carbohydrate utilized by parasitic helminths was, in no instance, converted to lactic acid. However, in the case of the tissue helminths, *Litomosoides carinii* and *Schistosoma mansoni*, lactic acid production accounts for at least 80 per cent of the total carbohydrate utilized anaerobically by these two organisms. This was established by isolation of the acid from media in which these organisms had been incubated and by subsequent preparation and identification of the benzimidazole and the p-bromophenacyl-derivatives (26, 27). The filariae form only L (+)-lactic acid (26) while the schistosomes produce racemic lactic acid (27). Only a small fraction of the anaerobically consumed carbohydrate is converted to acetic acid by *Litomosoides carinii* (26). Evidence also has been obtained that lactic acid is the major end product of a third tissue helminth, the nematode, *Dracunculus insignis* (28), which lives in the subcutaneous tissues of raccoons (33).

Pasteur (134) discovered that the rate of fermentation by yeast is considerably higher under anaerobic conditions than in the presence of air. Subsequent investigations have revealed that oxygen decreases the utilization of carbohydrate and suppresses or decreases the accumulation of the products of anaerobic metabolism in a large variety of animal tissues and bacteria (50). Twelve times more energy is

liberated from the oxidation of one mole of glucose to CO_2 and water than from its fermentation (11). Consequently, in organisms which possess mechanisms for the complete oxidation of carbohydrate and which are able to utilize the energy derived from this process, fermentations and other anaerobic reactions may be reduced greatly by the presence of oxygen. Thus, in most vertebrate tissues, anaerobic glycolysis[2] is much higher than aerobic glycolysis (218). In the free living worm, *Lumbricus terrestris*, glycogen consumption is six times lower under aerobic than under anaerobic conditions (118) indicating a high efficiency in the oxidative utilization of carbohydrate. That the earthworm has a primarily aerobic type of metabolism is revealed by the fact that it does not survive in an oxygen-free atmosphere for a period of more than 24 hours (118).

A survey of parasitic helminths, in which aerobic and anaerobic fermentations have been measured, reveals that the presence or absence of oxygen has little or no effect on fermentations in those organisms which have a predominantly anaerobic type of metabolism. Conversely, in worms whose dependence on respiratory metabolism has been established, a marked reduction of fermentation occurs in the presence of oxygen. The available evidence indicates that the large intestinal worms and the tissue helminth *Schistosoma mansoni* have a predominantly anaerobic type of metabolism (see above). In such organisms oxidative metabolism has little or no effect on their rates of fermentation. Alt and Tischer (5) and Von Brand (194) found no difference between the total amounts of acids formed by *Moniezia expansa* under aerobic and anaerobic conditions. Therefore, oxygen appears to have no inhibitory effect on the rate of fermentation of this organism, provided the acids formed anaerobically are the same as those produced aerobically. In *Ascaris lumbricoides* anaerobic production of valeric acid is twice as high as in air and anaerobic glycogen consumption is 1.2 to 1.3 times higher than that occurring aerobically (195). Evidently the presence of oxygen results in a significant, but relatively slight reduction of fermentation of this parasite. In *Parascaris equorum* this effect is still smaller. According to Toryu (183, 184), the production of valeric and lactic acids and the utilization of glycogen by this helminth are only 3 to 10 per cent lower in air than in nitrogen. Carbohydrate utilization and lactic acid production by *Schistosoma mansoni* are the same under anaerobic conditions and in atmospheres whose oxygen content varies between 1 and 100 per cent (25, 27).

On the other hand, pronounced differences between the rates of aerobic and anaerobic fermentation have been observed in two parasitic helminths whose dependence on oxidative metabolism has been established. Von Brand and Simpson (197, 208) have demonstrated that the larval nematode *Eustrongylides*, when kept anaerobically, utilized three times more glycogen than under aerobic conditions. Anaerobically, this parasite produces a considerable amount of acid but this does not occur in the presence of air (197, 203). Anaerobically, the filarial worm, *Litomosoides carinii* converts 80 per cent of the utilized carbohydrate to lactic acid, but under aerobic conditions only 30 to 45 per cent is converted (26). Inhibition of respiration

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[2]The term 'fermentation' is used for anaerobic metabolism of carbohydrate while 'glycolysis' is reserved for the production of lactic acid from carbohydrate, a special case of fermentation (see also Von Brand, 210).

by cyanide or cyanine dyes results in a compensatory increase in the rate of glycolysis of the filariae (26). Acetate production by this organism is 2.5 to 3 times higher aerobically than anaerobically (26).

The observations discussed above lead to the following tentative conclusion: The more a parasitic helminth is dependent upon anaerobic metabolism, the less are its fermentations reduced by oxygen. It appears that fermentation is markedly reduced by oxygen only if the organism can utilize substantial amounts of energy derived from oxidative metabolism. This ability seems to be lacking in worms which possess a predominantly anaerobic type of metabolism. In these organisms respiration and fermentation are independent of each other. This would explain why their rates of fermentation are not, or are only slightly, decreased regardless of whether or not the parasites respire, and why inhibition of the oxygen uptake of *Schistosoma mansoni* does not result in a compensatory increase of glycolysis. Therefore, parasitic worms could be classified into two groups: *1*) Those organisms in which respiratory metabolism has little or no effect on their rate of fermentation. This group does not appear to require or to utilize energy liberated by oxidative reaction. *2*) Those helminths which utilize oxidative energy. In these organisms inhibition of respiration results in an increase in fermentation and eventually in their death. Investigations of a larger number of parasitic helminths should determine the validity of such an hypothesis and classification.

Anaerobiosis of many parasitic helminths and of other invertebrates is followed by a temporary rise in the oxygen uptake after aerobic conditions are re-established (18, 181, 86, 70–72, 47, 48, 114, 203, 210, 211). This repayment of the oxygen debt incurred during anaerobiosis has been considered as a general phenomenon among invertebrates. However, the respiration rates of *Litomosoides carinii* and of *Schistosoma mansoni* are the same before and immediately following anaerobiosis (26, 27). It has been assumed that during anaerobiosis products of fermentation accumulate and that these are oxidized as soon as aerobic conditions are re-established (210). Such an interpretation would explain the absence of a post-anaerobic increase of the oxygen uptake of the filariae and of the schistosomes; the major product of fermentation of both organisms is lactic acid and addition of this substance to the medium produces no increase in their oxygen uptake. It has not been established which products of fermentation are oxidized by organisms in which a post-anaerobic increase of respiration occurs.

Little is known about the intermediary carbohydrate metabolism of parasitic helminths. Evidence has been obtained that carbohydrate metabolism of *Litomosoides carinii* is associated with phosphorylations. When this organism is incubated aerobically in a medium containing radioactive phosphorus as inorganic phosphate, incorporation of P^{32} in the organic acid soluble phosphate fraction is at least 3.5 times higher in the presence of glucose than in its absence (29). If aqueous extracts of *Ascaris lumbricoides* are incubated in the presence of fluoride esterification of inorganic phosphate can be demonstrated (151). Extracts of male *Schistosoma mansoni* exhibit high zymohexase and glyceraldehyde phosphate dehydrogenase activity (27). These observations indicate that lactic acid formation in these three parasites may proceed according to the Embden-Meyerhof scheme. Further work is necessary to establish

this with certainty. It also remains to be determined whether the various types of phosphatases, which have recently been found in a number of parasitic helminths (30, 135–137, 149), play a significant role in the carbohydrate metabolism of these organisms. In several respects the metabolism of pyruvic acid by *Litomosoides carinii* is similar to that of certain mammalian tissues and bacteria. Anaerobically the filariae convert two moles of pyruvate to one mole of lactate, one mole of acetate and one mole of CO_2 (26). This anaerobic dismutation of pyruvate also occurs in brain (225) and in a number of bacteria (107, 108). Aerobically, the filariae metabolize pyruvate to acetate, lactate, acetylmethylcarbinol (16), and to other as yet unidentified product or products. Suggestive evidence has been obtained indicating that oxidation of carbohydrate by small intestinal nematodes may involve the tricarboxylic acid cycle (152). In the latter organisms, in contrast to the filariae (26), oxidation of acetate occurs (152).

More than 45 years ago, Weinland (228) demonstrated the formation of valeric acid from glycogen by extracts from *Ascaris lumbricoides*. In addition, several statements in the literature refer to the 'glycolytic' activity of cell-free material originating from parasitic helminths (62, 116), indicating the presence of enzymes which catalyze the removal of glucose. While the remarkable advances in the field of intermediary carbohydrate metabolism of vertebrates, yeast and bacteria were initiated by the use of cell-free extracts, it is astonishing to note that similar methods have not been applied to parasitic helminths for the investigation of the intermediary reactions and the enzyme systems concerned with the conversion of carbohydrate to volatile and higher fatty acids.

LIPID METABOLISM

Experimental data obtained by chemical determination of lipids indicate that higher fatty acids are not metabolized to any significant degree by the large parasitic helminths inhabiting the intestinal tract and the bile ducts. Weinland (227) found that no decrease in the lipid content of *Ascaris* occurs when this organism is kept in a non-nutrient salt solution for several days. These observations were confirmed by Schulte (161) and by Von Brand (195). Subsequently, the latter author (201) demonstrated that starvation of *Ascaris* for five days under aerobic conditions results in a small loss of fat. However, at the end of the experimental period, the sum of the fat excreted with the eggs and of the fat of the *Ascaris* body was identical with the initial fat content of *Ascaris* (201). Similar experiments with *Fasciola hepatica* (192, 233) and with *Moniezia expansa* (194) have revealed that the sum of the lipid excreted and present in the worms after starvation was higher than the initial lipid content of the organisms. This increase can be explained by the fact that higher fatty acids are products of the carbohydrate metabolism of these two parasites (see preceding section). However, the fat content of the bodies of the helminths remained constant throughout the experimental period (233, 194). Mueller (127) has reported a decrease in the fat content of *Ascaris* tissue kept in the perienteric fluid of this organism for eight days. On the basis of this frequently quoted observation he concluded that fat oxidation occurs in *Ascaris*. In these tissue cultures, fat was not determined chemically, but only by histological staining techniques. Results obtained in this

manner are difficult to evaluate on a quantitative basis. Furthermore, even if the reduction in stainable fat reflected a true decrease in the lipid content of *Ascaris* tissue, this would not prove that oxidation of fat had occurred. It is quite possible that this material merely passed into the surrounding fluid.

Several parasitic helminths possess esterase activity since fatty acid esters of glycerol and acetylcholine are hydrolyzed by extracts from these organisms. Lipase and choline esterase activities of *Fasciola hepatica*, of *Taenia taeniformis* and of *Taenia pisiformis* were found to be much lower than those of free living platyhelminths (135). Lipase activity has been detected also in *Strongylus edentatus* (146), *Ascaris* (146) and in two acanthocephala (30); cholinesterase activity in *Parascaris equorum* (8), in *Taenia crassicolis* (7) and in *Dypylidium caninum* (7). The substrate specificity and the physiological significance of these esterases have not been determined.

Several lipids with unusual properties occur in parasitic helminths. Flury (62) found that the major portion of the unsaponifiable fraction of *Ascaris* consists of a wax-like material which had the characteristics of an alcohol of high boiling point and which he called 'ascaryl alcohol.' This was studied in greater detail by Schulz and Becker (162) who isolated it both from *Ascaris lumbricoides* and from *Parascaris equorum*. On the basis of the elementary analysis of the pure compound (melting point 84° C.), these authors ascribe to it the formula $C_{32}H_{64}O_4$. Only two of the oxygen atoms could be accounted for by hydroxyl groups. The other two participate in ether linkages. The exact structure of this interesting compound has not been elucidated. It is present in the eggs of *Parascaris equorum* (54), but it also has been isolated in large quantities from male *Ascaris lumbricoides* (209).

Lesuk and Anderson (120) isolated from *Cysticercus fasciolaris* a cerebroside which contains as its nitrogenous base dihydrosphyngosin, but no sphyngosin, the usual constituent of cerebrosides. The same authors found that fatty acids of lecithin isolated from this organism were completely saturated and consisted mainly of palmitic acid. Data on the phospholipid content of *Ascaris lumbricoides* have been obtained recently by Rogers (151). According to Smorodinzev and Bebechine (169) the lipids of *Taeniarhynchus saginatus* contain an extremely large amount of unsaturated fatty acids. Since the segments of this cestode partially disintegrate in the intestinal tract and since unsaturated fatty acids produce hemolysis, these authors suggest that absorption of these acids might cause the anemia observed in infestations with cestodes. This hypothesis appears to be supported by the experiments of Wardle and Green (223) who produced anemia by feeding the fatty acids isolated from two tapeworms, *Moniezia expansa* and *Diphyllobothrium latum*.

PROTEIN METABOLISM

Only a few investigations on the protein metabolism of parasitic helminths have been carried out. Weinland (232) found that the daily excretion of nitrogen by *Ascaris lumbricoides* amounts to 15 to 20 mg/100 gm. wet weight. This was confirmed by Von Brand (195). According to this author, nitrogen excretion was the same under aerobic and anaerobic conditions and approximately 30 per cent of the excreted nitrogen originated from eggs and egg shells. Weinland (232) showed that one-third of the excreted nitrogen consists of ammonia. The total nitrogen excreted by *Fasciola hepatica* (233) and by *Moniezia expansa* (194) also has been determined.

Urea does not appear to be a product of the protein metabolism of *Ascaris* or of other parasitic helminths. Chitwood (36) has found that 24 hours after *Ascaris* has been removed from its host, the excretory fluid of the parasite contained no urea. Ammonia, but neither urea nor uric acid, is present in *Fasciola hepatica*, *Moniezia benedini* and *Taenia pisiformis* (188). No urease, allantoinase or allantoicase activity could be detected in *Fasciola hepatica* (188, 61). Van Grembergen and Pennoit-de-Cooman (188) also have reported that extracts of *Moniezia benedini* and of *Taenia pisiformis* have no urease activity. On the other hand, these cestodes, as well as *Fasciola hepatica*, have a high arginase activity (188). According to Schopfer (160) uric acid is formed by *Cysticercus tenuicolus* because the liquor of the cyst contains 6.5 times more uric acid than the extracellular fluid of the host.

Extracts possessing proteolytic activity have been prepared from a great number of parasitic helminths (105, 179, 1, 62, 63, 116, 165, 154, 36, 191, 147, 135). Optimal proteolytic activities of such crude extracts were observed between *p*H 2.2 and 8.0 and varied with the organism. In some of them several *p*H optima were noted indicating the presence of more than one proteolytic enzyme (135). Since none of these enzymes has been purified to any significant degree no definite comparison with the properties of proteolytic enzymes from other animals is possible. Rogers (147) has demonstrated that the proteolytic activity of extracts from *Strongylus edentatus* is 5 to 8 times higher than that of extracts of *Ascaris*. The former nematode ingests blood and tissue while the latter receives its food supply from the intestinal contents in which proteins are already partially digested by the host.

Histochemical evidence has been reported indicating that ribonucleo-proteins are synthesized during fertilization of eggs of *Parascaris equorum* (133).

A characteristic property of cestodes is their low protein content (166 to 168). Kent and Macheboeuf (101 to 104) have observed interaction of protein fractions from the cestode *Moniezia expansa* with glycogen and with an alcohol-soluble material which gives a positive Pettenkoffer test. This color reaction is obtained with bile acids, a great number of other steroids (215, 156), other aromatic compounds (60) and with some aliphatic alcohols and acids (60). The nature of the interaction of proteins of *Moniezia* with glycogen and with other substances remains open to further investigations.

The external cortical layer of the cuticle of *Ascaris* has been found to consist of keratin (35). This protein was detected also in the hooks of *Cysticercus fasciolaris* (43). Chitin has been found to occur in the egg membranes of *Parascaris equorum* (53, 238), of *Ascaris lumbricoides* (37, 158), of pinworms (97), and of *Macracanthorhynchus hirudinaceus* (200).

RESPIRATORY ENZYMES

A number of investigations on the enzymatic mechanisms catalyzing the transfer of hydrogen in parasitic helminths have been carried out. Gourevitch (73) observed that *Fasciola hepatica* and *Parascaris equorum* the metabolism of which is predominantly anaerobic contain considerably less flavin than insects and the tissues of vertebrates. Since in bacteria the flavin concentration is much higher in anaerobic than in aerobic organisms (221) the author concluded that this rule does not apply to metazoa.

Circumstantial evidence is available that flavine enzymes are involved in the hydrogen transfer mechanisms in parasitic helminths (34, 3). Pennoit-de-Cooman and Van Grembergen (135) have demonstrated the enzymatic reduction of methylene blue by extracts of *Fasciola hepatica*, *Taenia pisiformis* and of *Moniezia benedini*. Reduction of methylene blue was greatly accelerated by the addition of succinate (135). Similar findings were made with *Ascaris lumbricoides* by Laser (114). Since flavin enzymes react with methylene blue it is possible that these enzymes catalyze the reduction of methylene blue observed in these experiments. They also indicate that the parasites studied possess high succinic dehydrogenase activity.

Laser has shown that, in the presence of atmospheric oxygen, enzymatic dehydrogenations in *Ascaris lumbricoides* result in the formation of hydrogen peroxide (114). This substance inhibited the respiration of the organism. Since the worms contain little catalase (123, 114), hydrogen peroxide accumulates when the nematodes are incubated in air. Removal of hydrogen peroxide by the addition of catalase resulted in an increase in the oxygen uptake of this organism (114). Extremely low catalase activity was found also in *Moniezia benedini*, *Taenia pisiformis* and *Cysticercus pisiformis* (135).

Observations of Harnisch (87) indicate that the body fluid of *Ascaris lumbricoides* and of several cestodes (*Moniezia expansa*, *Abothrium rugosum* and *Abothrium crassum*) contain one or several factors essential for optimal respiration of these organisms. According to this author the low oxygen uptake of washed tissue mince is greatly increased by the addition of the body fluid of the parasites while the body fluid alone has no respiratory activity. It would be of interest to determine whether the body fluid supplies substrates or a coenzyme activating respiratory mechanisms of these helminths.

Keilin (98) detected in *Ascaris lumbricoides* the spectral absorption bands of cytochrome C, A and B. Similarly, the approximate absorption bands of cytochrome C were present in *Diphyllobothrium latum* (67), *Triaenophorus lucii* (67), *Allassostoma magnum* (236), and *Camallanus trispinosus* (236), and those of cytochrome B and C were found in *Moniezia benedini* (187) and in *Fasciola hepatica* (189). Treatment of a tissue mince of *Moniezia benedini* with hydrosulfite, hydrogen peroxide or pyridine had the same effects on the spectral characteristics of this material as on those of cytochrome C and B (187). Similarities of the absorption spectra of the cytochromes with those found in the parasites do not necessarily prove that the latter contain the cytochrome system. The observed optical properties of the worms may merely indicate that these organisms contain some hematin-like pigments. Whether these pigments have the same enzymatic properties as the cytochromes has not been determined. As pointed out by Keilin and Hartree (100) the most fundamental property of cytochrome C is its activity as a biological oxidation catalyst. Since homogenates of filariae had no cytochrome C activity (27) when tested with cytochrome oxidase and hydroquinone (76), convincing evidence for the presence of cytochrome C in parasitic helminths is lacking.

Nor has it been demonstrated that a major portion of the hydrogen transport of any parasitic helminth is mediated by cytochrome oxidase. In fact, several observations indicate that this enzyme might not play a significant role in a number of these organisms.

The effect of inhibitors of cytochrome oxidase (99) on the respiration of parasitic worms has been studied. Respiration of the larvae of *Trichinella* is strongly inhibited by low concentrations of cyanide (171). High concentrations (2.5×10^{-3} to 1×10^{-2}M) of cyanide and azide inhibit the respiration of *Eustrongylides* larvae to an extent of 72 and 85 per cent, respectively, but with lower concentrations this effect is much less pronounced (207). A reduction of the oxygen uptake of *Diphyllobothrium latum* to 15 per cent of its control value was observed only with very high cyanide concentrations (5×10^{-3} to 1×10^{-2}M) (67, 68). Incomplete respiratory inhibition by high concentrations of cyanide was also observed in *Taenia taeniformis* (237), in *Moniezia benedini* (187) and in the following intestinal nematodes: *Ascaridia galli*, *Nematodirus Spp.* and *Nippostrongylus muris* (150). Respiration of *Neoaplactana glaseri* (150), of *Litomosoides carinii* (26) and of *Schistosoma mansoni* (27) was inhibited completely by considerably lower cyanide concentrations. In concentrations up to 1×10^{-2}M cyanide did not inhibit the oxygen uptake of *Ascaris lumbricoides* (114, 190). However, this apparent absence of inhibition might be due to the removal from the reaction mixture of oxaloacetic acid by cyanide through cyanohydrine formation since oxaloacetic acid inhibits the respiration of *Ascaris* (114). Reduction of the oxygen uptake of parasitic helminths by cyanide may merely indicate the presence of a heavy metal containing respiratory enzyme, but not necessarily that of cytochrome oxidase. This is illustrated by the following example: *Litomosoides carinii*, the respiration of which is more sensitive to cyanide than that of most other parasitic helminths, possesses no cytochrome oxidase activity since homogenates of this organism do not oxidize reduced cytochrome C (27), the specific substrate of cytochrome oxidase. While this enzyme is inactivated by carbon monoxide in the absence of light (220) respiration of *Moniezia benedini* is inhibited to a slight degree (187) and that of *Diphyllobothrium latum* is not affected at all under these conditions (67, 68). The fact that paraphenylenediamine increases the respiration of larval *Eustrongylides* (207) and of *Monziezia benedini* (187) does not prove the presence of the cytochrome-cytochrome oxidase system in these organisms, because, conceivably, this labile substance could also be oxidized by other enzymes. Since the respiration of *Ascaris* results in the formation of hyrogen peroxide (114), it does not appear likely that cytochrome oxidase is involved in the hydrogen transfer mechanisms of this parasite because reduced cytochrome oxidase reacts with atmospheric oxygen to form water and not hydrogen peroxide.

RESISTANCE TO DIGESTION

Parasitic helminths are not digested in the intestine despite an abundance of proteolytic enzymes in this habitat. Neither are living intestinal nematodes or cestodes digested by trypsin (32). However, rapid digestion occurs if these organisms have been killed (32). The mechanism by which intestinal helminths are protected from digestion may be similar to that which prevents the destruction of many other living cells (e.g., those of the gastro-intestinal mucosa) by proteolytic enzymes. Fermi (56) and Northrop (128) demonstrated that when living earthworms, arthropods, fish, or protozoa were placed in solutions of trypsin or pepsin, no digestion and no decrease in the enzyme content of the solution occurred. However, when killed by heat, the same organisms removed a considerable amount of these proteolytic en-

zymes and were rapidly digested. Northrop (128) concluded, therefore, that the membranes of living cells are not permeable to pepsin and trypsin.

In round worms this general impermeability of living cells to digestive enzymes is enhanced by the external cuticle which is resistant to digestion. This cuticle is particularly well developed in parasitic nematodes. As demonstrated by the observation of Filipjev (58) intra-vitam stains, e.g., methylene blue, penetrate the cuticles of free-living worms, but not those of *Ascaris*, the external cuticular layer of which consists of a keratin (62) that is not digested by trypsin or pepsin (35). The protective role of the cuticle against digestive enzymes was demonstrated in 1878 by Fredericq (66), who placed intact *Ascaris marginata* in pancreatic juice. No digestion occurred in this medium. However, when the parasites were cut in several fragments all tissues with the exception of the cuticle were digested rapidly. De Wale (49) made similar observations with living *Taenia saginata* which was digested by pepsin or trypsin only if a slight incision was made in the proglottis or the scolex. It is evident, therefore, that the cuticle of parasitic helminths is impermeable to digestive enzymes.

Another factor which may play an important role in the protection of parasitic helminths against digestion is the presence of trypsin and pepsin inhibitors in these organisms. This was discovered in 1903 by Weinland (230), who found inhibitors of the proteolytic activities of trypsin and of pepsin in extracts of *Ascaris* and of *Taenia*. The observations were confirmed by Fetterolf (57) and Tallquist (179) for tapeworms and by Hamill (78) for *Parascaris equorum*. The latter author showed also that the trypsin inhibitor of *P. equorum* is dialyzable, heat-stable in acid and neutral solutions, heat-labile in alkali and that it is precipitated only by high concentrations of alcohol. He concluded that the inhibitor is not a protein, but that its properties are similar to those of a polypeptide. Similar observations were made by Harned and Nash (79). Stewart (175) found that nematodes living in the gastrointestinal tract of sheep (46) contain a pepsin inhibitor. He ascribed the decreased digestion of proteins by lambs heavily infested with these parasites to the action of this inhibitor (174). According to Sang (154) the trypsin inhibitor also has the properties of a proteolytic enzyme. Since no extensive fractionation and purification of the inhibitor was attempted by this author, his findings may signify merely that the inhibitor had not been separated from proteolytic enzymes. The same difficulty was encountered by Von Bonsdorff (191), who failed to detect any inhibition of trypsin or of pepsin by extracts of *Diphyllobothrium latum* and of *Taenia saginata*. These extracts had marked proteolytic activity and, therefore, may have masked the presence of inhibitors when added to trypsin or pepsin. Furthermore, Von Bonsdorff (191) makes no statement as to how his extracts were prepared. As already noted by Weinland (230) and confirmed by Mendel and Blood (126), the trypsin and pepsin inhibitors of parasitic helminths are quite insoluble and can be extracted only by very thorough grinding. The purification and isolation of a trypsin inhibiting polypeptide from *Ascaris* by Collier (38) has supplied definite evidence for the existence of such a compound. It is similar in almost every respect to the trypsin inhibitors crystallized from beef pancreas by Kunitz and Northrop (112) and isolated from blood serum (55) by Schmitz (159). However, in contrast to the latter two substances, inhibition of trypsin activity by the polypeptide from *Ascaris* was not

reversed at a low *p*H (38). In view of these similarities, it would be of interest to determine whether the trypsin inhibitor of *Ascaris* is supplied by the host or whether it is synthesized by the parasite itself. Since the pepsin inhibitor present in parasitic helminths has not yet been purified, no comparison of its properties with those of the pepsin inhibitor crystallized by Herriot (129) from pancreas is possible.

In contrast to their marked anti-tryptic and anti-peptic activity extracts from parasitic helminths have no inhibitory action on papain (126). Living *Ascaris* are digested by this (15) and other proteolytic enzymes of vegetable origin such as bromellin (14), present in pineapple juice, and by ficin (217, 145, 216, 6) isolated from the sap of *Ficus laurifolia.*

It can be concluded, therefore, that the resistance of intestinal helminths to digestion may be produced by at least three factors: *1*) the impermeability of living cells to digestive enzymes; *2*) the impermeability of the external cuticle; and *3*) the action of trypsin and pepsin inhibitors of the parasites. At present, the relative significance of each of these factors for the protection of the organisms against digestion cannot be ascertained.

EFFECT OF ANTHELMINTIC AGENTS ON THE METABOLISM OF PARASITIC WORMS

Routine testing programs, in which large numbers of unrelated compounds have been assayed for anthelmintic activity, have constituted the principal approach to the experimental chemotherapy of helminthic infestations up to the present time. In the course of such empirical studies, little knowledge has been gained concerning the mechanism of action of existing anthelmintic agents and the basic physiology of the parasites involved, despite the great potential value that such information would have in suggesting new and more logical approaches to this problem. Attention has been called to this situation in a recent review by Wright (240). The fundamental question as to whether any anthelmintic drug in use at the present time exerts its effect by inhibiting essential metabolic processes of the parasites against which it is effective has not been investigated to any significant extent.

It has been shown recently, however, that two groups of compounds, possessing anthelmintic activity in infested animals, produce their effects by inhibiting certain metabolic activities of the parasites. Recent experimental evidence indicates that the marked chemotherapeutic effectiveness of the cyanines against the filariasis of the cotten rat is based on their inhibitory effect on the oxidative metabolism of *L. carinii* (26, 234, 138). The administration of cyanine dyes has little or no effect on the survival *in vivo* of *S. mansoni* (24, 25, 141) and of certain larger helminths inhabiting the intestinal and biliary tracts (77, 141). These refractory organisms exhibit little or no dependence on respiration (see above). The cyanines, however, do possess activity against *Ancylostoma caninum* (77), which is believed to have an essential requirement for oxidative metabolism (198, 199). Thus, inhibition of respiratory processes appears to be the basis for the chemotherapeutic action of the cyanines against *L. carinii* and *A. caninum*; at the same time, it probably explains the lack of a chemotherapeutic effect against worms whose metabolism is predominantly anaerobic.

By way of contrast, the rate of glycolysis of *S. mansoni* is inhibited by a number of naphthoquinones which, however, have little effect on the respiration of the trema-

todes (23–25). These compounds have slight but definite chemotherapeutic activity against *S. mansoni* in mice, because they potentiate the effect of very small and otherwise ineffective doses of 'Fuadin' (23, 24). The rate of glycolysis of schistosomes removed from mice treated with this combination is markedly reduced (23). These observations indicate that the chemotherapeutic activity of such naphthoquinones is based on their ability to decrease the rate of glycolysis of *Schistosoma mansoni*. The fact that this effect is markedly decreased by serum albumin explains (23, 25), at least in part, the low chemotherapeutic activity *in vivo*. 'Fuadin', a much more effective chemotherapeutic agent in this condition when large doses are employed, inhibits glycolysis of schistosomes to a lesser degree than oxygen uptake (24, 25). Thus, this compound is quite effective in reducing *aerobic* metabolism, which at best plays a minor role in the survival of the worms; inhibition of essential glycolytic processes, on the other hand, occurs only with concentrations of the drug approaching those capable of producing injury to the host. These findings parallel the well-known and regrettable fact that clinically effective doses of 'Fuadin' frequently produce toxic reactions in the human host. The reversal of the anti-metabolic effects of 'Fuadin' against *S. mansoni* by BAL (British Anti-Lewisite) suggests that the inhibitory effects of the antimonial on glycolysis are due to inactivation of sulfhydryl enzymes (27a). The enhancement of the anti-glycolytic effect of p-chloromercuric benzoate by BAL *in vitro* (27a) is an interesting paradox. The possibility that a combination of these two agents might exert a chemotherapeutic effect against *S. mansoni in vivo* is an intriguing one, especially because BAL reduces the toxicity of mercury for the mammalian host.

As demonstrated recently by Baldwin (10, 12), low concentrations of santonin, an effective agent against *Ascaris lumbricoides in vivo*, have a selective toxic action on the ganglion located in the 'nerve ring' of this worm, and hexylresorcinol, an equally effective but less toxic ascaricide, has a direct paralyzing effect on its muscle. Trim (185) demonstrated that hexylresorcinol readily penetrates the cuticle of the ascarids. Subsequently, several factors affecting the rate of diffusion of hexylresorcinol and of other phenols into nematodes have been studied (186, 148, 4). Since the action of the anthelmintics is, in some cases at least, a specific one, involving specific parts or organs of the worms, the question as to the mechanism by which these localized effects are produced presents itself.

The evidence discussed in this review indicates that the biochemical characteristics of parasitic worms vary greatly from one species to another. In view of these differences, which are much more pronounced than those existing among vertebrates, it is not surprising that many drugs which are highly effective against one particular type of parasite, are completely inactive against others. Even among parasitic worms possessing many morphological similarities, profound metabolic differences may prevail. This is well illustrated by the marked chemotherapeutic activity of cyanine dyes against the filarial worm, *Litomosoides carinii*, on the one hand (234, 26, 138), and their complete lack of activity against two other adult filarial species, *Dirofilaria immitis* (141) and *Wuchereria bancrofti* (155a). It was not possible to study the latter parasite, which resides in the lymphatic system of its human host, *in vitro*; hence the possibility exists that the cyanine dyes were ineffective because of their failure to enter the lymph following their intravenous injection into man.

In dogs, however, comparable doses of cyanine dyes administered by the same route bestowed on the lymph the ability to inhibit the respiration of *L. carinii in vitro* (140). *Dirofilaria immitis*, of course, was subjected to very high concentrations of cyanine dyes *in vivo*, since the drug was injected into the vascular system, the actual site of occurrence of the infestation, yet the parasite survived. The metabolic differences between *L. carinii* and *W. bancrofti* are reflected also in the fact that 1-diethylcarbamyl-4-methylpiperazine ('Hetrazan') possesses marked chemotherapeutic activity against the latter parasite *in vivo* (155), while its effect on the former was of a very low order, both *in vivo* and *in vitro* (91). In the case of yet another tissue nematode, *Dracunculus insignis*, low concentrations of the cyanines produce a considerable reduction of the oxygen uptake, but no compensatory increase in the rate of glycolysis (28). This suggests that *Dracunculus insignis* also is much less dependent on oxidative metabolism than is *Litomosoides carinii*.

Such profound biochemical variations may be of considerable importance with regard to the development of new anthelmintic drugs. They may afford opportunities to inhibit essential metabolic reactions of the parasite, which may not occur in the host, or which, if present, may not be essential to its functional integrity. Furthermore, since morphological and taxonomic similarities of parasitic worms do not necessarily indicate biochemical similarities of parasitic worms, the metabolic characteristics of the particular organism against which chemotherapy is directed must be investigated rather than that of related species of greater availability. Once the essential metabolic reactions of a parasite are known, attempts to block such processes may be initiated.

Up to the present time only catabolic reactions of parasitic helminths have been studied. As pointed out by Hotchkiss (95), the anabolic metabolism of pathogenic organisms may prove to be even more vulnerable to inhibition. Since such reactions proceed at relatively slow rates, the organisms must be cultured aseptically outside the host for prolonged periods of time. In the past 15 years considerable progress has been made in establishing such conditions with parasitic worms, although this has not been possible as yet with media of known chemical composition.[3] Once this latter goal has been achieved, a study of the exact nutritional requirements of parasitic helminths will be feasible. Furthermore, under such conditions, experiments can be designed, in which attempts are made to block the utilization of essential metabolites by competitive and non-competitive inhibitors. It is quite possible that the essential requirements of a given parasite for several factors may differ qualitatively and quantitatively from those of the host. Thus, under proper conditions of dosage, prevention or reduction of the utilization of such factors might injure the parasite, but not its host.

The concept of inhibiting the essential metabolic reactions of parasitic helminths, theoretical at the present time, will increase in feasibility as additional information is acquired in regard to the specific chemical reactions involved. The number of points of attack likewise will increase on this basis. It is to be expected, however, that inhibitors effective *in vitro* frequently may not be active *in vivo*, due to inacti-

[3] Since this problem is outside the scope of this review, the reader is referred to reviews on this subject by Hoeppli *et al.* (94), by Hobson (93), and by Smyth (170), and to recent work of Stephenson (172).

vation, unfavorable distribution, metabolic alteration, slow absorption, or rapid excretion by the host. In some instances, the host's ability to exert such influences may be decreased by chemical modification of the metabolic inhibitor in a way which does not alter the intrinsic activity of the compound *in vitro*, as well as its toxicity for the host. These latter factors represent large problems in themselves and practical results may not occur in the immediate future. Nevertheless, it is believed that advances in the field of comparative biochemistry of parasitic worms can form the basis for a more rational approach to the chemotherapy of helminthiases, an approach which should supplement, and eventually may even replace the presently prevailing empirical methods.

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DETERMINATION OF AMINO ACIDS BY MICROBIOLOGICAL ASSAY[1]

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IT WAS REPORTED in September 1943, by Kuiken *et al.* (79) of the Texas Agricultural Experiment Station that nine amino acids (cystine, glutamic acid, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, and valine) were essential for the growth of *Lactobacillus arabinosus* 17-5 and that three amino acids (isoleucine, leucine and valine) could be determined rapidly and accurately by microbiological techniques similar to those utilized for vitamin assays. In December of that year these workers (80) described their microbiological procedure and reported the results of assays of gelatin and four food products for these three amino acids. The basal medium employed was that developed by Snell and Wright (162) in 1941 for the determination of nicotinic acid modified to contain *p*-aminobenzoic acid, a tomato juice eluate, and 17 amino acids in place of acid-hydrolyzed casein. During the intervening years microbiological methods have been elaborated in the laboratories of Lyman, Stokes, Snell, Elvehjem and other investigators for the determination of nearly all of the naturally occurring amino acids. Many of the amino acids have been determined in purified proteins, foods, blood, urine, sweat, spinal fluid, hormones, enzymes, viruses, bacteria and bacterial toxins, fungi, plant products and other types of biological materials. It is gratifying, therefore, that, through microbiology, significant advances have been made in our knowledge of amino acids and proteins in biology and medicine.

Achievements of such magnitude and apparent value are not uncommon in the annals of biological science, and, as in comparable cases, there is a background of notable accomplishments by many earlier workers without which present successes would have been impossible. That the assay potentialities of micro-organisms, virtually unknown a decade ago either for vitamins or amino acids, have been revealed in this relatively short time is of course only the culmination of countless experiments performed since microbes were first observed by von Leeuwenhoek (83) in 1685. We may be reminded, however, that modern investigations were not initiated until about 1860 by Pasteur who discovered that bacteria are causal agents of fermentation, putrefaction and disease.

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[1] Snell (146, 153, 155) has published excellent review articles on microbiological methods for the estimation of amino acids. Doctors M. N. Camien and L. B. Rockland have rendered valuable assistance in the preparation of this paper, which was presented essentially in the present form on August 9, 1948 at the Gordon Research Conference, New London, New Hampshire. Dr. Clayton D. Sander, Eastern States Farmers' Exchange, Buffalo, N. Y., rendered valuable assistance in checking the data and calculations. The author is indebted to the Nutrition Foundation for financial aid in support of his microbiological research work and for payment of extra publication costs. Paper 50. For Paper 49, see Murphy and Dunn, in press.

During several decades Pasteur, Liebig and other outstanding investigators of the time concerned themselves with the metabolism of yeasts, particularly fermentation, in attempting to find a chemical basis for the physiological activity of the cell. Because of the theoretical and practical importance of yeasts and fermentative processes, such studies have been carried on increasingly since Pasteur's day by many workers mentioned in reviews of these topics by Harden (52) and other authors (1, 58, 84, 106, 108). Following the isolation of *Bacillus anthracis* about 1850, the bacterial forms responsible for scarlet fever, meningitis, botulinism, tetanus, tuberculosis and other diseases were identified prior to 1900. Nencki (107) and other investigators, referred to in reviews by Leach (82) and other authors (18, 140, 175), have determined the chemical composition of microbial cells in the search for clues to the causes of diseases and the pattern of evolutionary processes. Extensive morphological and physiological studies have been made, also, leading to the classification

TABLE 1. COMPOSITION OF BASAL MEDIA EMPLOYED BY PASTEUR AND OTHER EARLY WORKERS

CONSTITUENT	BASAL MEDIUM FOR YEAST		BASAL MEDIUM FOR *B. typhosus* AND OTHER ORGANISMS
	Pasteur, 1860	Wildiers, 1901	Uschinsky, 1893
Sucrose	X	X	
Ammonium tartrate or lactate	X		X
Yeast ash	X		
Glycerol			X
NaCl or KCl		X	X
$CaCl_2$ or $CaCO_3$		X	X
$MgSO_4$		X	X
K_2HPO_4 or Na_2HPO_4		X	X
Sodium aspartate			X
NH_4Cl		X	

of bacterial types as given in the Bergey's *Manual of Determinative Bacteriology* (6) and other treatises.

The elaboration of synthetic media on which bacteria will grow has been a problem of major concern even to the earliest workers. The conclusion of Pasteur (116) in 1860 that yeasts could be cultured on an aqueous solution containing only chemically-defined substances was unacceptable to Liebig and, apparently, was disproved by Wildiers (178) in 1901. The latter worker found that yeast would not grow unless an unknown factor, which he named 'bios'[2] in addition to sucrose and the salts shown in table 1 was present in the culture medium. On the other hand, Uschinsky (172) had shown earlier (1893) that typhoid, cholera, diphtheria and certain other organisms could be cultivated on a synthetic medium of the composition shown in table 1. Of interest in this connection is the recent report of Wooley and Sebrell (184) of a microbiological procedure for the determination of tryptophan with a particular strain of typhoid bacillus grown on a synthetic basal medium containing

[2] The 'bios' problem has been reviewed by Tanner (171).

besides 15 amino acids only glucose, sodium chloride, magnesium sulfate and potassium dihydrogen phosphate.

During the past half century numerous workers interested primarily in the metabolism of microorganisms have grown bacteria on synthetic media varying little in composition from the mixtures employed by Uschinsky. Types of organisms which have been cultured include *Escherichia coli* (36, 44, 141), *Corynebacterium diphtheriae* (171), *Bacillus lactis aerogenes* (56), *Streptococcus faecalis* (145), *Bacillus pyocyaneus* (78), *Mycobacterium tuberculosis* (10, 22, 78, 86, 88, 135, 136, 143, 169), *Azotobacter chroococcum* and other strains (12, 13, 48, 75) of Azotobacter, and various related bacterial forms. Since it has been observed that certain bacteria grow poorly, if at all, on media of composition as simple as that of the original or modified Uschinsky formula, improvement in the nutritional quality of culture media has been an objective of numerous workers since the time of Pasteur. Natural materials or extracts of naturally-occurring products found to be effective supplements of synthetic media include yeast, blood, peptones, beef muscle, liver, fish, urine, manure, and grass. Owing to the complexity of such mixtures slow progress was made until recently in determining the identity of any components which might be essential for the growth of bacteria.

The bacteria of most importance to the present discussion are the Lactobacillus genus of the Lactobacilleae and the Streptococcus and Leuconostoc genera of the Streptococceae. These non-motile, non-spore forming, Gram-positive cocci and rods are essentially anaerobic organisms which produce lactic acid, and in some cases other organic acids, from carbohydrates. These bacteria are of practical importance because of their prominent role in the manufacture of sauerkraut, the production of silage, the aging of cheese, the brining of olives and pickles, the spoilage of beer, the decay of the teeth and other processes. The names of some common types of lactic acid bacteria are given in table 2.

It was first observed in 1857 by Pasteur (115) that lactic acid is produced by bacteria which ferment sugar and the first true lactic acid bacterium, *Streptococcus lactis*, was isolated in 1873 by Lister (85) who employed a basal medium essentially the same as that (table 1) described by Pasteur (116) in 1860. Most early workers, including Kayser (64) in 1894, grew bacteria on media containing peptonized milk, peptones, gelatin, yeast or other protein supplement and the standard types of media shown in table 3 were suggested by Henneberg (55) in 1903.

The most fundamental studies of the lactic acid bacteria made by early workers were those of Orla-Jensen who initiated (110) his investigations on the nutrition, morphology and physiological characteristics of micro-organisms in Copenhagen about 1898. Orla-Jensen's classical treatise (111) entitled, "The Lactic Acid Bacteria," was published in 1919 and his book on dairy bacteriology (112) in 1931. Of particular interest in relation to the topic under discussion are his studies (113) in 1936 on growth factors in milk and peptones and on the vitamin and nitrogen requirements of lactic acid bacteria. It was established that unknown factors are required for the growth of *bulgaricus*, *helveticus*, *casei*, *lactis* and other Lactobacilli and it was concluded that riboflavin and probably pantothenic acid are essential-growth substances for these organisms. Advantage of these discoveries was soon taken by other work-

ers in elaborating 'synthetic' diets for lactic acid bacteria containing these two vitamins. This milestone in nutritional research could not have been reached easily at an earlier time even though the vitamin concept had been accepted as valid since the experiments of Hopkins (59) in 1912. Although the presence of water-soluble substances in milk which promote the growth of young rats was noted by Osborne

TABLE 2. HISTORY OF DISCOVERY OF COMMON LACTIC ACID BACTERIA[1]

ORGANISM	DISCOVERER	DATE
Streptococcus lactis	Lister	1873
Leuconostoc mesenteroides	Cienkowski; Van Tiegham	1878
Lactobacillus casei	von Freudenreich	1890
Lactobacillus lactis	Leichmann	1896
Lactobacillus delbrueckii	Leichmann; Lafar	1896
Lactobacillus acidophilus	Moro	1900
Lactobacillus fermenti	Beijerinck	1901
Streptococcus faecalis	Thiercelin	1902
Lactobacillus buchneri	Henneberg	1903
Streptococcus salivarius	Andrewes-Horder	1906
Leuconostoc dextranicum	Beijerinck	1912
Lactobacillus plantarum	Orla-Jensen	1919
Lactobacillus pentoaceticus	Fred-Peterson-Davenport	1919
Leuconostoc citrovorum	Hammer	1920
Lactobacillus arabinosus	Fred-Peterson-Davenport	1921
Lactobacillus pentosus	Fred-Peterson-Davenport	1921
Lactobacillus lycopersici	Mickle	1924

[1] Data taken from Bergey *et al.* (6).

TABLE 3. HENNEBERG'S[1] MEDIA FOR CULTURING LACTIC ACID BACTERIA

CONSTITUENT	MEDIUM		
	1	2	3
	%	%	%
Glucose	5.0	5.0	5.0
Peptone	1–5	1.0	1.0
Yeast infusion		1.0	
Prune infusion			1. 0
KH_2PO_4	0.8		
$MgSO_4$	0.1		

[1] Henneberg (55).

and Mendel (114) in 1913, it was not until 1926 that thiamine (63), 1933 that riboflavin (77) and 1940 that pantothenic acid (180) were available in crystalline form.

A most notable program of research on the nutrition of lactic acid bacteria was inaugurated in 1919 by Fred and Peterson (41–43, 60, 117, 123–132, 144, 165) at the University of Wisconsin. Their collaborators during succeeding years were Bohonos, Hutchings, Snell, Strong, Tatum, Wood, Woolley, Wright and other investigators who also have become eminent in this field. Although for more than a decade these

workers were concerned, primarily, with the fermentative products and processes of lactic acid and other bacteria cultured on yeast extract or cereal grain infusions, they identified and named *Lactobacillus pentoaceticus* (43), *Lactobacillus arabinosus* (42) and *Lactobacillus pentosus* (42).

In 1937 Snell, Tatum and Peterson (161) investigated the growth of *Lactobacillus delbrueckii* on a basal medium essentially the same as that employed in 1923 by Speakman (163) of the University of Toronto in his studies on the fermentation of glucose and other sugars by *Bacillus granulobacter pectinovorum*. The composition of Speakman's medium is given in table 4 and that of Snell *et al.* is shown as *medium 1* in table 5. Growth supplements employed by the latter workers included acid-hydrolyzed casein, tryptophan and an extract of potato or liver. The same year Snell, Strong and Peterson (159) made extensive studies on the nutrition of thirteen species of lactic acid bacteria. The basal medium (*medium 2*, table 5) was the same as *medium 1* except for the omission of the casein hydrolyzate and the addition of sodium acetate, cystine, riboflavin, and a supplement of liver, yeast or whey extract.

TABLE 4. BASAL MEDIUM USED BY SPEAKMAN[1] FOR FERMENTATION OF SUGARS BY BACILLUS GRANULOBACTER PECTINOVORUM

CONSTITUENT	PER CENT	GLUCOSE OR OTHER CARBOHYDRATE	PER CENT
Glucose or other carbohydrate	3.0	$MgSO_4$	0.02
Peptone	0.5	NaCl	0.001
KH_2PO_4	0.05	$FeSO_4$	0.001
K_2HPO_4	0.05	$MnSO_4$	0.001

[1] Speakman (163).

Riboflavin was incorporated into the medium because of Orla-Jensen's (113) report the preceding year that it was an essential growth factor for lactic acid bacteria.

The first thoroughly practicable microbiological procedure for the determination of any vitamin (riboflavin) was described by Snell and Strong (158) in 1939. The basal medium (*medium 3*, table 5) was the same as *medium 2* except for the omission of tryptophan and riboflavin. Analogous procedures for the determination of pantothenic acid and nicotinic acid, respectively, were reported by Pennington, Snell and Williams (121) in 1940 and by Snell and Wright (162) in 1941. The basal media were *media 4* and *5* in table 5. The basal medium utilized previously was altered markedly for use in the nicotinic acid assay by omitting peptone and adding tryptophan, adenine, guanine, uracil, riboflavin, pantothenic acid, pyridoxine, thiamine, an acid hydrolyzate of casein, and a concentrate of biotin. Riboflavin and pantothenic acid were determined with *Lactobacillus casei* and nicotinic acid with *Lactobacillus arabinosus* 17-5. These authors introduced pyridoxine and biotin into their basal medium because Möller (94, 95) had reported in 1938 and 1939 that these vitamins were essential or stimulatory for lactic acid bacteria. Pantothenic acid was added because the suggestion of Orla-Jensen (113) that it, in addition to riboflavin, was required by lactic acid bacteria had been corroborated by Snell, Strong and Peterson (160) in 1939.

In 1946, Roberts and Snell (138) described a procedure considered to be suitable for the determination of five vitamins (biotin, folic acid, nicotinic acid, pantothenic acid and riboflavin) with *Lactobacillus casei* and a single basal medium. The medium

TABLE 5. BASAL MEDIA EMPLOYED BY SNELL ET AL. FOR VITAMIN ASSAYS
(Values given as gm. %)

CONSTITUENT	MEDIUM					
	1 1937	2 1937	3 1939	4 1940	5 1941	6 1946
Glucose	1.0	1.0	1.0	1.0	1.0	2.0
Sodium acetate:		0.6	0.6	0.6	0.6	2.0
Peptone	0.5	0.5	0.5	0.5		
Casein, enzymatic digest	5[1]			0.2[1]	0.5[1]	2.0
K_2HPO_4	0.05	0.05	0.05	0.05	0.05	0.25
KH_2PO_4	0.05	0.05	0.05	0.05	0.05	0.25
$MgSO_4 \cdot 7H_2O$	0.02	0.02	0.02	0.02	0.02	0.08
NaCl	0.001	0.001	0.001	0.001	0.001	0.004
$FeSO_4 \cdot 7H_2O$	0.001	0.001	0.001	0.001	0.001	0.004
$MnSO_4 \cdot 3H_2O$	0.001	0.001	0.001	0.001	0.001	0.016
Tryptophan	0.015	0.01			0.01	
Cystine		0.01	0.01	0.01	0.01	0.01
Adenine sulfate, mg. %					1	1
Guanine·HCl, mg. %					1	1
Uracil, mg. %					1	1
Riboflavin, γ %		10		10	20	50
Ca pantothenate, γ %					10	50
Nicotinic acid, γ %					20	50
Pyridoxine·HCl, γ %					20	50
Thiamine·HCl, γ %					10	50
p-Aminobenzoic acid, γ %						10
Biotin, γ %					Concen.	0.4
Folic acid, γ %						0.2
Supplement	S	S[1]	S[2]			

S, Liver or potato extract. S[1], Liver, yeast or whey extract. S[2], Yeast extract.
[1] Acid hydrolyzate.

Col. 1. Snell, E. E., E. L. Tatum, and W. H. Peterson. *J. Bact.* 33: 207, 1937. Nutrition of *L. delbrueckii*.
Col. 2. Snell, E. E., F. M. Strong and W. H. Peterson. *Biochem. J.* 31: 1789, 1937. Nutrition of 13 lactic acid bacteria,
Col. 3. Snell, E. E. and F. M. Strong. *Ind. Eng. Chem., An. Ed.* 11: 346, 1939. Determination of riboflavin with *L. casei*.
Col. 4. Pennington, D., E. E. Snell and R. J. Williams. *J. Biol. Chem.* 135: 213, 1940. Determination of pantothenic acid with *L. casei*.
Col. 5. Snell, E. E. and L. D. Wright. *J. Biol. Chem.* 139: 675, 1941. Determination of nicotinic acid with *L. arabinosus* 17-5.
Col. 6. Roberts, E. C. and E. E. Snell. *J. Biol. Chem.* 163: 499, 1946. Determination of riboflavin and folic acid and standard curves for nicotinic acid, pantothenic acid and biotin with *L. casei*.

(*medium 6*, table 5) was identical in composition with that (*medium 5*, table 5) employed for the determination of nicotinic acid with *L. arabinosus* 17-5 except that an enzymatic digest of casein was substituted for acid-hydrolyzed casein, tryptophan was omitted, crystalline biotin was substituted for a biotin concentrate, and *p*-aminobenzoic acid and crystalline folic acid were added. The most drastic changes were

marked increases in the concentration of glucose, sodium acetate, casein digest, buffer and other salts, and vitamins.[3]

A summary of the data on the requirements of 13 lactic acid bacteria[4] for 8 vitamins taken from recent publications by Shankman *et al.* (148) and by Snell (157) is given in table 6. Although the 6 organisms investigated in both laboratories were shown to have nearly the same vitamin requirements, from 1 to 2 organisms were found by Snell, but not by Shankman *et al.*, to require *p*-aminobenzoic acid, biotin, folic acid, thiamine and pyridoxine. A possible explanation for this difference is that the basal medium employed by Shankman *et al.* contained more nutrients, many at higher concentrations than other media utilized previously in studying the nutritional

TABLE 6. VITAMINS REQUIRED BY LACTIC ACID BACTERIA[1]

ORGANISM	*p*-AMINO-BENZOIC ACID	BIOTIN	FOLIC ACID	NICO-TINIC ACID	PANTO-THENIC ACID	RIBO-FLAVIN	THIA-MINE	PYRI-DOXINE
L. arabinosus 17-5[2]	(X)	X		X	X			(X)
L. brassicae		X		X	X		X	
L. buchneri		X		X	X	X		
L. casei[2]		X	(X)	X	X	X		X
L. delbrueckii LD-5[2]		X	(X)	X	X	X		X
L. fermenti 36[2]		(X)		X	X			
L. gayonii				X	X			
L. lycopersici		X		X	X	X	X	
L. mannitopoeus		X		X	X		X	
L. mesenteroides P-60[2]	(X)	X		X	X		(X)	(X)
L. pentoaceticus			X	X	X		X	
L. pentosus		X		X	X			
S. fecalis R		X	X	X	X			
No. vitamins essential	0	10	2	13	13	4	4	2

[1] Data taken from Shankman *et al.* (148) and Snell (157).

[2] Organism studied by Shankman *et al.* and Snell. All others studied only by Shankman *et al.*

(X) Vitamin considered essential by Snell only.

requirements of lactic acid bacteria. As shown in table 7 this basal medium contained 21 amino acids, 12 vitamins, 3 buffer and 5 other salts, 3 purines, 1 pyrimidine and 1 carbohydrate. It seems of particular interest that on this relatively complete medium, *p*-aminobenzoic acid was not required for the growth of any of the 13 organ-

[3] Microbiological procedures for the determination of riboflavin, pantothenic acid, biotin, nicotinic acid, pyridoxin, inositol, thiamin, and folic acid with *L. casei*, *Saccharomyces cerevisiae*, *L. arabinosus* 17-5, and *S. faecalis* (*lactis* R) were described in 1941 by Williams and co-workers (179). The content of these vitamins in various tissues of the rat, mouse, cow, and pig, and in milk, urine, and commercial animal rations was determined by these investigators.

[4] Excellent reviews on the nutrition of lactic acid and other bacteria have been presented by Burrows (14), Clifton (21), Henneberg (55), Kluyver (67), Knight (71, 72), Koser and Sanders (76), McIlwain (91), Orla-Jensen (111), Peskett (95), Peterson and Peterson (133), Snell (154, 156, 157), Stephenson (164) and Werkmann and Wood (176).

isms listed in table 6 whereas folic acid was essential for only 2 organisms (pentoaceticus and fecalis R) and pyridoxine for only one (casei).[5]

Many types of biological materials have been assayed for vitamins by microbiological methods since 1939 and the data obtained have been of inestimable value in assessing the nutritional quality of rations and foods. It is recognized, however, that many of the data have only relative significance and that there are inherent difficulties which must be overcome before microbiological assays for vitamins in foods can be made with the degree of precision and accuracy attained by the best chemical methods in the analysis of natural products for some biological constituents. It may be mentioned in this connection that the vitamins for which microbiological

TABLE 7. BASAL MEDIUM EMPLOYED BY DUNN ET AL. IN STUDY OF LACTIC ACID BACTERIA NUTRITION[1]

CONSTITUENT	mg. %	CONSTITUENT	mg. %	CONSTITUENT	mg. %
DL-Alanine	66.7	DL-Serine	66.7	$MnSO_4 \cdot 4H_2O$	1
Asparagine, natural	66.7	DL-Threonine	66.7	NaCl	35
L-Arginine·HCl	66.7	DL-Tryptophan	66.7	Thiamine·HCl	0.1
L-Cysteine·HCl	66.7	L-Tyrosine	66.7	Pyridoxine	0.16
L-Glutamic acid	66.7	DL-Valine	66.7	Pyridoxamine·2HCl	0.01
Glycine	66.7	Adenine sulfate·$2H_2O$	1.4	Pyridoxal·HCl	0.01
L-Histidine·HCl·H_2O	66.7	Guanine·HCl·$2H_2O$	1.4	DL-Ca pantothenate	0.2
L-Hydroxyproline	66.7	Uracil	1.2	Riboflavin	0.2
DL-Isoleucine	66.7	Xanthine	1.2	Nicotinic acid	0.2
L-Leucine	66.7	Glucose (gm.)	2.0	Biotin	0.0005
DL-Lysine·HCl	66.7	Sodium acetate (gm.)	1.2	*p*-Aminobenzoic acid	0.01
DL-Methionine	66.7	NH_4Cl (gm.)	0.6	Folic acid (conc.)	0.0005
DL-Norleucine	66.7	KH_2PO_4	50	Choline chloride	1
DL-Norvaline	66.7	K_2HPO_4	50	Inositol	2.5
DL-Phenylalanine	66.7	$MgSO_4 \cdot 7H_2O$	20		
L-Proline	66.7	$FeSO_4 \cdot 7H_2O$	1		

[1] Dunn (34).

procedures are recommended by the Association of Vitamin Chemists (3) in their 1947 book entitled, *Methods of Vitamin Assay*, include only riboflavin, nicotinic acid and thiamine.[6]

It should be apparent from the foregoing discussion that the development in 1943 of microbiological methods for the determination of amino acids was the logical outcome of the extensive earlier studies on bacteriological media, bacterial metabolism, vitamins and amino acids. Various strains of lactic acid bacteria had been isolated and were readily maintained in pure culture. Reasonably well-defined basal media

[5] That *L. delbrueckii* LD-5 is the same as *L. casei* has been reported by Rogosa (139) and Dunn *et al.* (34).

[6] Microbiological procedures for the determination of thiamin, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, inositol, biotin, and folic acid, have been described by various workers in *Estimation of the Vitamins* by Dann and Satterfield (23).

which supported good growth of these organisms were available. Many of the B-complex vitamins had been isolated in crystalline form. Microbiological techniques, applicable to the determination of amino acids, had been developed for vitamin-assay purposes. Each of the 19 amino acids known to occur in proteins was obtainable in purified form either as the natural L-antipode or the synthetic DL-mixture.

Considerable time elapsed, however, after it was known that bacteria utilize amino acids before it was discovered that they could be employed to determine amino acids. More than 50 years ago aspartic acid and asparagine were added to basal media to stimulate the growth of microorganisms. In 1893 Uschinsky (172) cultivated diphtheria bacilli, and in 1911 Lowenstein and Pick (88) grew tubercle bacilli, on synthetic media containing aspartic acid. This practice[7] has been followed by other workers (2, 22, 65, 135, 143, 169) interested in growing the tubercle bacillus. Since 1922 basal media containing up to 19 amino acids have been employed in nutritional studies of numerous types of bacteria including Corynebacterium (97, 98, 100–103), Salmonella (15, 39, 87), Clostridium (20, 37, 38, 40, 68, 104, 105, 168, 183), Streptococcus (66, 89, 90, 96, 185), Staphylococcus (46, 69, 70, 177), Brucella (74), Pasteurella (24), Escherichia (36, 141) and other genera (10, 47, 118, 174).

As shown in table 8 it has been demonstrated, following the pioneer investigations of Koser and Rettger (75) in 1919, that *Eberthella typhosa* (119), *Corynebacterium diphtheriae* (97, 98, 100), *Clostridium sporogenes* (40), *Bacillus anthracis* (47), *Streptococcus hemolyticus* (90, 185), *Streptococcus faecalis* (145) and other organisms can be cultivated successfully on synthetic media. It was shown, furthermore, that from 1 to 6 amino acids were essential for the growth of each of these strains. Advantage has been taken of the assay potentialities of *Proteus morganii* by Pelczar and Porter (119, 120) in their microbiological method for the determination of pantothenic acid and, probably, it would have been possible to devise comparable procedures for the determination of tryptophan and several other amino acids. As shown in figure 1, the standard curves which Müeller (99) obtained in 1935 denoting the growth of diphtheria bacilli at different levels of cystine, methionine, glutamic acid or tryptophan resemble those used a decade later for the determination of these amino acids.

Orla-Jensen (113) found from his classical experiments reported in 1936 that *L. casei*, *L. helveticus* and other Lactobacilli could be cultured in a synthetic medium containing 17 amino acids provided a supplement of whey was added equivalent to one fourth of the total nitrogen in the complete medium. The growth response of these organisms was measured in terms of titratable acid produced. The next year Wood, Anderson, and Werkman (181) at Iowa State College cultivated *L. delbrueckii* and 5 other lactic acid bacteria in a basal medium containing in addition to 17 amino acids glucose, sodium acetate, ammonium sulfate and other inorganic salts, thiamine, riboflavin, and yeast extract. In 1939, Möller (95) grew *L. plantarum* on an analogous basal medium. Highly significant studies on the nutrition of *L. buchneri*, *L. lycopersici*, and *L. mannitopoeus* were reported by Wood, Geiger and Werkman (182) in 1940. These organisms were cultured successfully in a nearly-synthetic amino acid-containing basal medium and the standard curves shown in figure 2 were ob-

[7] The literature prior to 1930 on use of synthetic media for the growth of diphtheria bacilli has been reviewed by Mayer (92).

TABLE 8. SYNTHETIC MEDIA USED TO GROW BACTERIA (1919–1941)

CONSTITUENT	KOSER AND RETTGER 1919	FILDES *et al.* 1933	MUELLER 1934	FILDES AND RICHARDSON 1935	GLADSTONE 1939	MCILWAIN 1940	WOOLLEY AND HUTCHINGS 1940	KOSER *et al.* 1941	SCHUMAN *et al.* 1941
Glucose	x	x			x	x	x	x	x
Liebig's extract			x						
Ash from hay				x					
Sodium citrate		x							x
Iron salt			x		x	x			
NH_4Cl		x				x			
NaCl	x	x	x				x	x	x
$MgSO_4$	x	x	x		x	x	x	x	x
$CaCl_2$	x		x					x	
KH_2PO_4	x	x	x	x	x	x	x		x
K_2HPO_4	x	x	x	x	x	x		x	x
Alanine		x		x	x	x	x	x	x
Arginine		x		$\bar{x}$	x	x	x	x	$\bar{x}$
Aspartic acid	x	x		x	x	x	x		x
Cystine		x	$\bar{x}$	x	$\bar{x}$	x	x	x	x
Glutamic acid	x	$\bar{x}$	$\bar{x}$		x	x	$\bar{x}$	x	$\bar{x}$
Glycine	x	x	x	x	$\bar{x}$	x	x	x	x
Histidine	x	$\bar{x}$	x	x	x	x	x	x	x
Hydroxyproline					x	x	x	x	x
Isoleucine				x	x	x	x		x
Leucine		x		$\bar{x}$	x	x	x	x	x
Lysine	x	x		x	x	x	x	x	x
Methionine			$\bar{x}$	x	x	x	x	x	$\bar{x}$
Phenylalanine		x	x	$\bar{x}$	x	$\bar{x}$	x	x	x
Proline		x			x	x	x	x	x
Serine				x		x	x	x	x
Threonine					x	x	x	x	x
Tryptophan	x	$\bar{x}$	$\bar{x}$	$\bar{x}$	x	x	$\bar{x}$	x	$\bar{x}$
Tyrosine	x	$\bar{x}$		$\bar{x}$	x	x	x	x	$\bar{x}$
Valine	x	$\bar{x}$	x	x	x	x	x	x	$\bar{x}$

$\bar{x}$—signifies amino acid essential for growth of organism.

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KOSER, S. A. AND L. F. RETTGER. *J. Infect. Dis.* 24: 301, 1919. Medium contained taurine, creatine and allantoin; 24 non-lactic acid organisms were tested.

FILDES, P., G. P. GLADSTONE, AND B. C. J. G. KNIGHT. *Brit. J. Exper. Path.* 14: 189, 1933. Organism tested was *Eberthella typhosus.*

MUELLER, J. H. *Proc. Soc. Exper. Biol. & Med.* 32: 318, 1934, and *J. Bact.* 29: 515, 1935; 30: 513, 1935. Supplements tested were thiamine, pantothenic acid and extracts of liver, muscle and heart. Diphtheria bacillus was the organism tested.

FILDES, P. AND G. M. RICHARDSON. *Brit. J. Exper. Path.* 16: 326, 1935. Organism tested was *Clostridium sporogenes.*

GLADSTONE, G. P. *Brit. J. Exper. Path.* 20: 189, 1939. Organism tested was *Bacillus anthracis.*

MCILWAIN, H. *Brit. J. Exper. Path.* 21: 25, 1940. Supplements tested were ornithine, citrulline, β-hydroxyglutamic acid, glucosamine, inositol, thiochrome, pyridoxine, pantothenic acid, β-alanine, riboflavin, betaine, and hydrolyzates of casein, yeast and egg albumin. Organism tested was *Streptococcus hemolyticus.*

WOOLLEY, D. W. AND B. L. HUTCHINGS. *J. Bact.* 39: 287, 1940. Basal medium contained Na_2SO_4, reduced iron, riboflavin, pyridoxine, and a pantothenic acid concentrate. Organism tested was *Streptococcus zymogenes* strain H-6905 (Lancefield Group D).

KOSER, S. A., B. B. BRESLOV, AND A. DORFMAN. *J. Infect. Dis.* 69: 114, 1941. Supplements tested were nicotinamide, coenzyme I, thiamine, cocarboxylase, β-alanine, Ca pantothenate, pyridoxine, riboflavin, inositol, glutamine, adenine, sodium pyrophosphate, biotin concentrate and traces of iron, manganese, copper and calcium salts. Organisms tested were members of the Brucella group.

SCHUMAN, R. L. AND M. A. FARRELL. *J. Infect. Dis.* 69: 81, 1941. Basal medium contained riboflavin, pantothenic acid and pyridoxine. Organism tested was *Streptococcus faecalis* (Lancefield Type D).

tained for 12 amino acids. Although relatively little acid was produced, owing primarily to the low concentration of amino acids in the medium, it is evident that probably it would not have been difficult to adapt the procedures of Wood *et al.* to the microbiological determination of amino acids.

As was stated earlier in this discussion the *L. arabinosus*-microbiological assay procedures of Kuiken *et al.* (79, 80) for the determination of isoleucine, leucine and

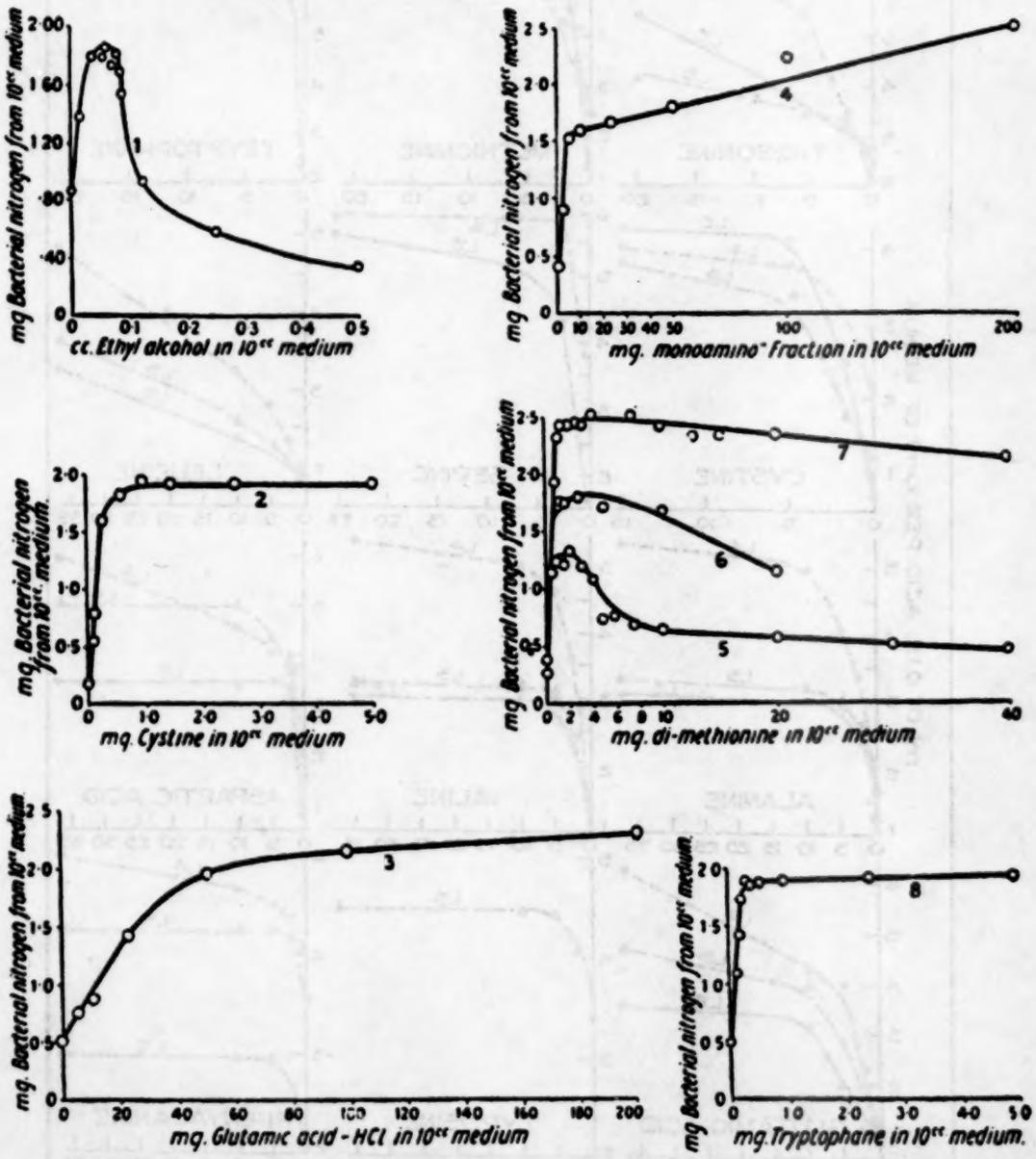
mg Bacterial nitrogen from 10cc medium
cc. Ethyl alcohol in 10cc medium
mg. monoamino acid fraction in 10cc medium
mg. Cystine in 10cc medium
mg. di-methionine in 10cc medium
mg. Glutamic acid - HCl in 10cc medium
mg. Tryptophane in 10cc medium

Fig. 1. STANDARD CURVES for 4 amino acids based on acid produced by C. diptheriae. (MUELLER, J. H. *J. Bact.* 29: 522, 1935).

valine were the first to be reported. The composition of the basal media employed in 1943 by Greene and Black (49) for the determination of tryptophan with *L. arabinosus* and by Shankman *et al.* (150, 151) for the determination of several amino acids with *L. arabinosus* and *L. casei* is given in table 9. It may be noted that these basal media were patterned after those formulated about the same time by Landy and Dicken (81) for the determination of six vitamins with *L. casei* and by Pollack

and Linder (134), Bohonos *et al.* (7), Hutchings and Peterson (62), Shankman (147), Smith (152) and Gaines and Stahley (45) for studies on the nutrition of *L. arabinosus*, *L. casei*, *L. pentosus*, *L. delbreuckii*, *Leuconostoc mesenteroides*, and *S. fecalis* R (*lactis*).

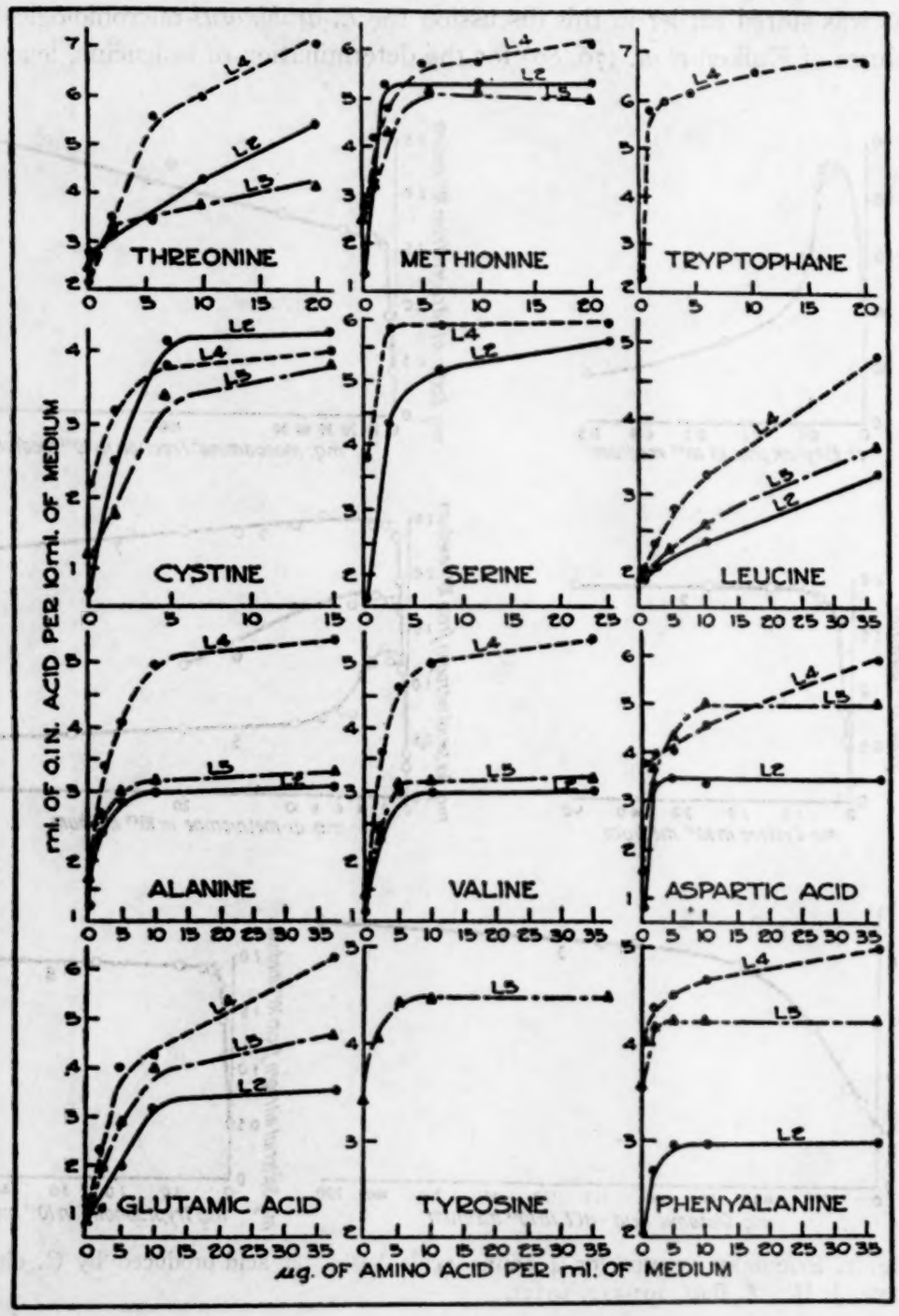

Fig. 2. STANDARD CURVES for 12 amino acids based on acid produced by L. buchneri, L. lycopersici and L. mannitopoeus (WOOD, GEIGER AND WERKMAN. *Ia. State Coll. J. Sci.* 14: 367, 1939–40).

As shown in table 9, changes made in the basal media during the past five years include the addition of ammonium sulfate, ammonium chloride, sodium citrate, succinic acid, inositol, choline, folic acid, pyridoxamine, pyridoxal, xanthine, as-

paragine, glycine, hydroxproline, norleucine and norvaline. Most workers have increased the concentration of glucose from 1 to 2 per cent, and in a few instances to 3 (26, 28) and even 4 per cent (173), to promote greater acid production and, ostensibly, to increase the convenience and accuracy of the titrations. The concentration of sodium acetate has been increased from 0.6 per cent to as much as 2.4 per cent (173) to facilitate greater acid production without significant decrease in *p*H. This advantage is offset somewhat by the increased difficulty with which the color change of the bromthymol blue indicator is detected in the visual titration procedure. Sodium citrate has been substituted for sodium acetate (137, 142) and both buffer salts have been introduced into some basal media (54) because of "the superiority of citrate over acetate in promoting growth and acid production (of *S. fecalis*) . . ." and "its (sodium acetate's) demonstrated growth-stimulating activity for several lactic acid bacteria. . . ." KH_2PO_4 was omitted and the concentration of the other usual salts was increased from 2- to 10-fold in the 1948 medium of Henderson and Snell (54). The concentration of the vitamins has been increased 10-fold, or even more, by most of the later investigators. The concentration of adenine, guanine and uracil has also been increased considerably by many workers. In general, nearly all of the basal media have contained about the same concentrations of amino acids as those employed initially by Kuiken *et al.* (79).

It may be noted from table 10 that all of the 19 amino acids, except hydroxyproline, which occur commonly in proteins, have been determined by microbiological methods in various natural materials. Each of the 18 amino acids has been determined by from 1 to 4 of the 5 organisms which have been employed commonly for this purpose. Three amino acids have been determined with *L. fermenti* 36, 10 each with *L. arabinosus* 17-5 and *L. casei*,[5] 14 with *S. fecalis* R and 18 with *Leuconostoc mesenteroides* P-60. That no assay procedure has been found for hydroxyproline is explained by the failure to discover any organism for which this amino acid is a growth-essential. Its uniqueness in this respect may indicate that hydroxyproline is synthesized by the lactic acid bacteria which have been studied or that it is not an essential metabolite.

The extreme fastidiousness of *L. mesenteroides* P-60 for amino acids was observed in 1944 by Dunn *et al.* (35) who obtained the standard curves shown in figure 3. Although hydroxyproline, norleucine and norvaline were found to be inactive, 18 other amino acids appeared to be growth promoters. Later workers have taken advantage of the assay potentialities of *L. mesenteroides* P-60 in determining all but one of the 18 amino acids for which standard curves have been reported. As shown in table 11, food proteins, corn steep liquor, urine, enzymes, viruses, bacterial toxins and other materials have been assayed for these amino acids with this organism in the laboratories of Barton-Wright (4, 5), Buehler (11), Cardinal and Hedrick (19), Dunn (29–32, 149), Heller and Kirch (53), Hier (57), Jones (61), Knight (73), Sauberlich and Baumann (142), Snell (50, 54), and Velick and Ronzoni (173). The experiments reported by Dunn *et al.* (34) in 1947 strengthened the view that assays for 15 amino acids might be made satisfactorily with *L. mesenteroides* P-60. It seemed doubtful, however, that alanine, proline or serine could be determined accurately, especially in foods and other complex mixtures of nutrients, since none of

TABLE 9. COMPOSITION OF BASAL MEDIA USED TO DETERMINE AMINO ACIDS WITH LACTIC ACID BACTERIA

(Values given as percentage)

CONSTITUENT	1942			1943									1944			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Glucose, gm.	1.0	2.0	1.0	2.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	1.75	2.0	2.0
$(NH_4)_2SO_4$, gm.													0.3	0.3		
NaAc, gm.	0.6[y]	1.2	0.6	0.6	0.6	0.6	0.6	0.6[x]	0.6	0.6	1.45	0.6	0.6	0.875	0.6	2.0
NH_4Cl, gm.																
KH_2PO_4, gm.	0.05	0.1	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.1	0.05	0.05	0.05
K_2HPO_4, gm.	0.05	0.1	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.1	0.05	0.05	0.05
$MgSO_4 \cdot 7H_2O$, gm.	0.02	0.04	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
NaCl, gm.	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
$FeSO_4 \cdot 7H_2O$, gm.	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
$MnSO_4 \cdot 4H_2O$, gm.	0.00067	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Inositol, γ		500											250			
Choline chloride, γ													250			
Thiamine·HCl, γ	10	20			10	10	10	10			10	10	10	40		20
Pyridoxine·HCl, γ	40	20		20	10	10	10	10		20	10	10	30	40	10	20
Ca-Pantothenate, γ	20	20	20	20	10	10	10	10		20	10	10	10	40	10	20
p-Aminobenzoic acid, γ					1	S	1				0.05		30	40	10	10
Riboflavin, γ	20	20	10	20	20	20	20	20		20	20	20	10	40	10	40
Nicotinic acid, γ	20	20	20	20	20	20	20	10		20	40	20	10	40	10	20
Biotin, γ	0.5	0.04		0.01	0.04	0.004	0.04	0.1		0.01	0.04	0.04	0.02	0.04	0.04	0.02
Folic acid, γ	0.05	0.5				S		0.005		1.0			1		0.025	
Adenine sulfate, mg.	0.5	1		2	1	1	1	1		2	1	1	1	0.4	1	1
Guanine·HCl, mg.	0.5	1			1	1	1	1			1	1	1	0.4	1	1
Uracil, mg.	0.5	1			1	1	1	1			1	1	1	0.4	1	1
Xanthine, mg.	0.5	1						1					1	0.4		
Alanine, mg.		1*		20*	20*	20	20*			10*	20*		20*	8.3*		20*
Aspartic acid, mg.		1		20*		40					40*		40	8.3*		
Asparagine, mg.	25	1			40		40			10*						40
Arginine·HCl, mg.		1		20	5	20	5	8		10	20		10	8.3		5

Cystine, mg.	10	1	10	20	10	20	10		10	10	20	10	10	8.3	10	10
Glutamic acid, mg.		†		20*	40	40	40	50*		10*	40*		100	8.3		40
Glycine, mg.		1		20									10			
Histidine·HCl·H_2O, mg.		1		20	5	20	5	4		10	20		10	8.3		5
Hydroxyproline, mg.		1		20									10	8.3		
Isoleucine, mg.		1*		20*	20*	20	20*	10*		10*	20*		20*	8.3*		20*
Leucine, mg.		1		20*	20	20	20	10*		10*	20*		10	8.3		10
Lysine·HCl, mg.		1		20*	20	20	20	20*		10*	20		10	8.3		20
Methionine, mg.		1*		20*	10*	20	10*			10*	20*		20*	8.3*		10*
Norleucine, mg.				20*									20*	8.3*		
Norvaline, mg.													20*			
Phenylalanine, mg.		1*		20*	10*	20	10*			10*	20*		20*	8.3*		10*
Proline, mg.		1		20		20					20		10	8.3		
Serine, mg.		1*		20*		20				10*	20*		20*	8.3*		
Threonine, mg.		1*		20*	20*	20	20*			10*	20*		20*	8.3*		20*
Tryptophan, mg.	10	1	10	20	3.3	20	3.3		10	10	20		10	8.3		4
Tyrosine, mg.		1		20	3.3	20	3.3			10	20*		10	8.3		4
Valine, mg.		1*		20*	20*	20	20*	16*		10*	20*		20*	8.3*		20*
Casein (acid hydrolyzate), gm.	0.5		0.5						0.5			0.5			0.25	
Organism	C	A-C F-P	A-C D-F M-P	C	A	A	A	F	M′	C	A	A	C	A	F	A

CONSTITUENT	1944				1945											
	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Glucose, gm.	1.0	2.0	2.0	1.0	1.66	2.0	1.0	2.0	2.0	2.0	1.75	1.0	2	1	2	1
$(NH_4)_2SO_4$, gm.											0.3	0.3				
NaAc, gm.	0.6	1.2	1.2	0.6	1.3		1.0[y]	1.2	0.725	0.6	0.875	0.6	1[y]	0.6[y]	1.2	0.6
NH_4Cl, gm.		0.6	0.6												0.6	
KH_2PO_4, gm.	0.05	0.05	0.05	0.05	0.03	0.45	0.05	0.054	0.05	0.05	0.05	0.1	0.05	0.05	0.05	0.05
K_2HPO_4, gm.	0.05	0.05	0.05	0.05	0.03		0.05	0.054	0.05	0.05	0.05	0.1	0.05	0.05	0.05	0.05
$MgSO_4 \cdot 7H_2O$, gm.	0.02	0.02	0.02	0.02	0.013	0.004	0.02	0.0216	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02

TABLE 9.—*Continued*

CONSTITUENT	1944				1945											
	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
NaCl, gm.		0.001	0.001	0.001	0.00066		1.0		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
$FeSO_4 \cdot 7H_2O$, gm.	0.001	0.001	0.001	0.001	0.00066		0.001	0.00108	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
$MnSO_4 \cdot 4H_2O$, gm.	0.001	0.001	0.001	0.001	0.00066		0.001	0.00108	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Inositol, γ												250				
Choline chloride, γ												250				
Thiamine·HCl, γ	100	100	100	100	13		50	40	10		40	50	100	100	100	20
Pyridoxine·HCl, γ	160	160	160	20	13		50	64	10	20[x]	40	1000	200	200	160	40[d]
Ca-pantothenate, γ	200*	200	200	20	26		10	80	10	20	40	20	100	100	200	20
p-Aminobenzoic acid, γ	10	0.01	0.01	20	13		1	4	0.05		40	30	50	50	0.01	4
Riboflavin, γ	200	200	200	20	26		20	80	20	20	40	20	100	100	200	20
Nicotinic acid, γ	200	200	200	40	26		50	80	40	20	40	20	100	100	200	20
Biotin, γ	0.05	0.5	0.5	0.5	0.06		1	0.2	0.04	0.01	0.04	0.1	0.5	0.5	0.5	0.02
Folic acid, γ	0.4	0.2	0.2	S						0.1		1.0			0.2	0.2
Adenine sulfate, mg.	10	1.2	1.2	5	0.66		1	1.2	0.5	2	0.4	1	0.5	5	1.2	1
Guanine·HCl, mg.	10	1.2	1.2	5	0.66		1	1.2	0.5		0.4	1	0.5	5	1.2	1
Uracil, mg.	10	1.2	1.2	5	0.66		1	1.2	0.5		0.4	1	0.5	5	1.2	1
Xanthine, mg.											0.4	1	0.5	5		
Alanine, mg.	20*	200*	200*	20*		11.9*			20*	20*	8.3*	20*		20*	200*	20*
Aspartic acid, mg.				20*		17.7*			40*	20*	8.3*	40		20*		20*
Asparagine, mg.	20	40	40												40	
Arginine·HCl, mg.	5	8	8	20		5.2			20	20	8.3	10		20	8	20
Cystine, mg.	10	12	12	20	6.6	6	10	12	20	20	8.3	10	10	20	12	20
Glutamic acid, mg.	†	15	15	20		9.8	†		†[b]	20*	8.3	100[n]		20	15	20*
Glycine, mg.	10	10	10	20		10				20	8.3	10		20	10	20
Histidine·HCl·H_2O, mg.	5	2	2	20		5			20	20	8.3	10		20*	2	20
Hydroxyproline, mg.	10	10	10	20		10.9				20	8.3	10		20	10	20
Isoleucine, mg.	20*	15*	15*	20*					20*	20*	8.3*	20*		20*	15*	20*
Leucine, mg.	20	7.5	7.5	20		17.5*			20*	20*	8.3	10		20	7.5	20*
Lysine·HCl, mg.	20*	16*	16*	20*		4.6			20	10	8.3	10		20	16*	10

Methionine, mg.	10*	4*	4*	20*		7.5			20*	20*	8.3*	20*		20*	4*	**20***
Norleucine, mg.	10*	10*	10*	20*						20*	8.3*	20*			10*	20*
Norvaline, mg.		10*	10*									20*			10*	
Phenylalanine, mg.	10*	6*	6*	**20***		8.2			20*	20*	8.3*	20*		20*	6*	**20***
Proline, mg.	10	2.5	2.5	20		7.7			20	20	8.3	10		20	2.5	20
Serine, mg.	5*	8*	8*	**20***					20*	**20***	8.3*	20*		20*	8*	20*
Threonine, mg.	20*	45*	45*	**20***					20*	20*	8.3*	20*		20*	45*	**20***
Tryptophan, mg.	5	1	1	**20**		†	10	†	20	20	8.3	10	†	†	1	**40***
Tyrosine, mg.	2	3	3	**20**		4.5			20*	20	8.3	10		20	3	**20**
Valine, mg.	20*	15*	15*	**20***		15.6			20*	20*	8.3*	20*		20*	15*	**20***
Casein (acid hydrolyzate), gm.					0.66		0.5	0.666					0.5			
Organism	A	M	M	A C	A	T	A	A	A	D	A	A M	A	A	M	F D

CONSTITUENT	1945								1946							
	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
Glucose, gm.	2	2	2	3	2	1	3	1	2	2[a]	2	2	1	2	2	2
$(NH_4)_2SO_4$, gm.										0.3	0.3				0.6	0.6
NaAc, gm.	1.2	1.2	2	1	1.2	0.6	1	0.6	2	2	2	1.2	0.6	1.2	1.2	1.2
NH_4Cl, gm.	0.6		0.6	0.3	0.6		0.3					0.6		0.6		
KH_2PO_4, gm.	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	1.6	0.4
K_2HPO_4, gm.	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	1.6	0.4
$MgSO_4 \cdot 7H_2O$, gm.	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.04	0.04
NaCl, gm.	0.001	0.001	0.001		0.001	0.001		0.001	0.001	0.5	0.5	0.5	0.5	0.001	0.002	0.002
$FeSO_4 \cdot 7H_2O$, gm.	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.0002	0.0002	0.0002	0.0002	0.001	0.002	0.002
$MnSO_4 \cdot 4H_2O$, gm.	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.002
Inositol, γ															250	250
Choline chloride, γ															250	250
Thiamine·HCl, γ	100	100	20	100	100	100	100	20	10	10	20	100	20	100	50	50
Pyridoxine·HCl, γ	160	160	20	160	160	160	160	20	10	20	20	160	120	160	1000	1000
Ca-pantothenate, γ	200	200	20	200	200	200	200	20	10	20	20	100	20	200	20	20
p-Aminobenzoic acid, γ	0.01	0.01	10	10	10	10	0.01	20	10	10	10	10	4	0.01	30	30
Riboflavin, γ	200	200	20	200	200	200	200	20	20	20	40	200	20	200	20	20
Nicotinic acid, γ	200	200	20	200	200	200	200	40	20	40	40	200	60	200	20	20

TABLE 9.—*Continued*

CONSTITUENT	1945								1946							
	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
Biotin, γ	0.5	0.5	.04	0.5	0.5	0.5	0.5	0.5	20	.05	0.05	0.5	0.04	0.5	0.1	0.1
Folic acid, γ	0.2	0.6		0.2	0.2	0.2	0.2	S				0.1	0.2	0.2	1.0	1.0
Adenine sulfate, mg	1.2	1.2	1	2.4	1.2	1.0	2.4	0.05	1	1	1	1.2	1	1.2	2	2
Guanine·HCl, mg	1.2	1.2	1	2.4	1.2	1.0	2.4	0.05	1	1	1	1.2	1	1.2	2	2
Uracil, mg	1.2	1.2	1	2.4	1.2	1.0	2.4	0.05	1	1	1	1.2	1	1.2	2	2
Xanthine, mg				2.4			2.4			1	1	1.2	1		2	2
Alanine, mg	400*	20*	20*	40*	266*	20*	100*	20*			20*	100*	20*	100*	40*	40*
Aspartic acid, mg											80*	80*	20*	80*	80	80
Asparagine, mg	80	20	40	20	53	40	50	20								
Arginine·HCl, mg	16	20	5	16	11	5	40	20			5	25	20	25	60	20
Cystine, mg	24	27.5 (HCl)	10	20	16	5[p]	50	20	20	20	10	10	20	10	20	20
Glutamic acid, mg	30	20	40	24	20	40	60	20			40	50	20	50	200	200
Glycine, mg	20		10	20	13		50	20				10	20	10	20	20
Histidine·HCl·H_2O, mg	4	20	5	9	3	5	9	20			5	10	20	10	20	20
Hydroxyproline, mg	20		10	20	13		50	20							20	20
Isoleucine, mg	30*	20*	20*	40*	20*	20*	100*	20*			20*	20*	20*	20*	40*	40*
Leucine, mg	15	20	20*	20	10	20	50	20			10	10	20	10	20	20
Lysine·HCl, mg	32*	20	20	20	21*	20	50	20*			40*	25*	20*	25*	20	20
Methionine, mg	8*	20*	10*	20*	5*	10*	50*	20*			10*	10*	20*	10*	40*	40*
Norleucine, mg	20*		10*	20*	13*		50*	20*				10*	20*	10*	40*	40*
Norvaline, mg	20*			20*	13*		50*					10*	20*	10*	40*	40*
Phenylalanine, mg	12*	†	10*	20*	8*	10*	50*	20*			10*	10*	20*	10*	40*	40*
Proline, mg	5		10	20	3.3		50	20				20*	20*	10	20	20
Serine, mg	16*	30*	5*	20*	11*		50*	20*				10*	20*	10*	40*	40*
Threonine, mg	90*	20*	20*	20*	60*	20*	50*	20*			20*	50*	20*	50*	40*	40*
Tryptophan, mg	2	20	4	3.2	1.3	3.3	8	20	10	†	8*	10*	60*	10*	20	20
Tyrosine, mg	6	20	4	10	4	3.3	25	20			4	10	20	10	20	20
Valine, mg	30*	20*	20*	40*	20*	20*	100*	20*			20*	20*	20*	20*	40*	40*
Casein (acid hydrolyzate), gm.									0.5	0.6						
Organism	M	C	A	F	M	A	F	A-C-F	A	A	A	M	F	M	M	F

CONSTITUENT	1946																
	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
Glucose, gm.	2	2	2	2	2	2	2	2	2	2	1	2	2	2	1	1	1
$(NH_4)_2SO_4$ gm.																	
NaAc, gm.	2	2.5[x]	2	2	2.5[x]	2	2	2	0.3[e]	1.2	0.6	2[y]	2	1.2	0.6	0.6[f]	0.6[f]
NH_4Cl, gm.				0.3	0.3					0.6				0.6			
KH_2PO_4, gm.	0.05		0.05	0.05		0.05	0.05	0.05	0.05	0.05	0.05		0.05	0.05	0.05	0.05	0.05
K_2HPO_4, gm.	0.05	0.5	0.05	0.05	0.5	0.05	0.05	0.05	0.05	0.05	0.05	0.25	0.05	0.05	0.05	0.05	0.05
$MgSO_4 \cdot 7H_2O$, gm.	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
NaCl, gm.	0.001	0.001	0.001	0.001		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.3	0.001	0.001	0.001
$FeSO_4 \cdot 7H_2O$, gm.	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
$MnSO_4 \cdot 4H_2O$, gm.	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Inositol, γ	250	250	250														
Choline chloride, γ	250	250	250														
Thiamine·HCl, γ	50	50	50	50	50	50	50	50	20	100	100	20	20	100	100	100	100
Pyridoxine·HCl, γ	500[h]	500[h]	500[h]	100	100	100	100	100	40[j]	200	20	120[k]	20	160	20	20	100[d]
Ca-pantothenate, γ	50*	50*	50*	50*	50*	50*	50*	50*	20	200	20	40	20	200*	20	20	20
p-Aminobenzoic acid, γ	50	50	50	10	10	10	10	10	0.1	0.01	20	1	10	0.01	20	20	20
Riboflavin, γ	50	50	50	50	50	50	50	50	50	200	20	20	40	200	20	20	20
Nicotinic acid, γ	100	100	100	100	100	100	100	100	100	200	40	60	20	200	40	40	40
Biotin, γ	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.5	0.05	0.04	0.02	0.5	0.5	0.5	0.5
Folic acid, γ	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.5	0.15	0.2	0.2		0.2	S	S	S
Adenine sulfate, mg.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.5	1.0	5	1.0	1.0	1.2	5	5	5
Guanine·HCl, mg.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.5	1.0	5	1.0	1.0	1.2	5	5	5
Uracil, mg.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.5	1.0	5	1.0	1.0	1.2	5	5	5
Xanthine, mg.		1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.5			1.0			5	5	5
Alanine, mg.	20*	10*	20*	20*	10*	20*			20*		4*			200*	40*	40*	40*
Aspartic acid, mg.						†			40*		12*						
Asparagine, mg.	40	40	40	20	20	**20**								40	40	40	40
Arginine·HCl, mg.	5	5	10	**5**	5	10			20		4.8[q]			8	**20**	20	**20**
Cystine, mg.	10	20	20	10	20	20	10	**10**	20	10	20	20	20	12[p]	20	20	20

Table 9.—*Continued*

CONSTITUENT	1946																
	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
Glutamic acid, mg.	40	40	40	40[n]	40[n]	40			40*		42*			15	40	40	40
Glycine, mg.		2	10	2	2	10		10	20		20			10	20	20	20
Histidine·HCl·H_2O, mg.	5	5	10	5	5	10			20		2.7			2	20	20	20
Hydroxyproline, mg.											1			10	20	20	20
Isoleucine, mg.	20*	20*	20*	20*	20*	20*			20*		2.5*			15*	20*	20*	20*
Leucine, mg.	20*	20*	20*	20*	20*	20*			20*		20*			7.5	20	20	20
Lysine·HCl, mg.	20	20	20	20	20	20		10	20		15*			16*	20*	20*	20*
Methionine, mg.	20*	10*	20*	10*	10*	20*		20*	†	†	5			4*	20*	20*	20*
Norleucine, mg.											6*			10*	20*	20*	20*
Norvaline, mg.														10*			
Phenylalanine, mg.	10*	10*	10*	10*	10*	10*			20*		8*			6*	20*	20*	20*
Proline, mg.			5	5	5	5		5	20		7			2.5	20	20	20
Serine, mg.		5*	20*	5*	5*	20*		10*	20*		12*			8*	20*	20*	20*
Threonine, mg.	20*	20*	20*	20*	20*	20*			20*		9*			45*	20*	20*	20*
Tryptophan, mg.	5*	10*	10*	5*	10*	10*	5*	10*	10	5	10	20	†	1.0	20	20	20
Tyrosine, mg.	5	10	10	5	10	10		10	20*	10*	6.5			3	20	20	20
Valine, mg.	20*	20*	20*	20*	20*	20*			20*		12*			15*	20*	20*	20*
Casein (acid hydrol.), gm.	0.2[r]	0.5[r]	0.5[r]				0.5	0.5[r]		0.75[s]		0.5	0.5				
Organism	A	F	M	A F	F	M	A F	M A-F	F	M	A	C	A	M	A C-F	F	F

CONSTITUENT	1947													1948	
	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
Glucose, gm.	2	2	2	2	1	1	2	2	2	2	2	2	2	2	2
$(NH_4)_2SO_4$, gm.													0.6		
NaAc, gm.	0.3[t]	2	1.2	1.2	0.6	0.6	0.3[t]	0.3[t]	2	2		1.2	2	0.1[f]	1.2
NH_4Cl, gm.			0.6	0.6					0.3			0.6		0.3	0.4
KH_2PO_4, gm.	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05			0.05	0.05		0.055

K_2HPO_4, gm.	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.5	0.5	0.05	0.05	0.5	0.055
$MgSO_4 \cdot 7H_2O$, gm.	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.08	0.02	0.02	0.08	0.022
NaCl, gm.	0.001	0.001	0.035	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.004	0.01
$FeSO_4 \cdot 7H_2O$, gm.	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.004	0.0011
$MnSO_4 \cdot 4H_2O$, gm.	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.0128	0.0011
Inositol, γ			2500												
Choline chloride, γ			1000												
Thiamine·HCl, γ	20	10	100	100	100	100	20	20	50	50	50	100	20	100	111
Pyridoxine·HCl, γ		200	160[m]	160	20	20[d]	40[d]	40[d]	20	20	20	160	20	20[u]	180
Ca-Pantothenate, γ	20	10	200	200	20	20	20	20	50*	50*	50*	200	20	100	220
p-Aminobenzoic acid, γ	0.1	10	10	0.01	20	20	0.1	0.1	10	10	10	0.01	10	20	11
Riboflavin, γ	20	20	200	200	20	20	50	50	50	50	50	200	40	100	220
Nicotinic acid, γ	40	50	200	200	40	40	100	100	100	100	100	200	20	100	220
Biotin, γ	0.1	0.04	0.5	0.5	0.5	0.5	0.1	0.1	0.1	0.1	0.1	0.5	0.02	1.0	0.55
Folic acid, γ	0.5	0.5	0.5	0.2	0.2	0.2	0.5	0.4	1	1	1	0.2		1.0	
Adenine sulfate, mg.	0.5	1	1.4	1.2	5	5	0.5	0.5	1	1	1	1.2	1	1.0	1.45
Guanine·HCl, mg.	0.5	1	1.3	1.2	5	5	0.5	0.5	1	1	1	1.2	1	1.0	1.37
Uracil, mg.	0.5	1	1.2	1.2	5	5	0.5	0.5	1	1	1	1.2	1	1.0	1.32
Xanthine, mg	0.5		1.2				0.5	0.5	1	1	1			1.0	
Alanine, mg.	100*		66.7*	400*	4*	4*	20*		20*			267*	20*	100*	46*
Aspartic acid, mg.	40*				12*	12*	40*							100*	
Asparagine, mg.			66.7	80					40			53	40		4.6
Arginine·HCl, mg.	20		66.7	16	20.8	4.8	20		10			10.7	5	20	46
Cystine, mg.	20	20	66.7[p]	24	20	20	20	10	†	†	†	16	10	10	9.2
Glutamic acid, mg.	20		66.7	30	47*	47*	20		40			20	[z]	100	92
Glycine, mg.	5		66.7	0.3	20	20	20		10			13.3		10	4.6
Histidine·HCl·H_2O, mg.	20		66.7	4	2.7	2.7	†		10			2.7	5	10	4.6
Hydroxyproline, mg.			66.7	20	1	1						13.3			
Isoleucine, mg.	20*		66.7*	30*	2.5*	2.5*	20*		20*			20*	20*	20*	46*
Leucine, mg.	20*		66.7	15	20*	20*	20*		20*			10	10	10	†
Lysine·HCl, mg.	20*		66.7*	32*	15*	15*	20		20			21.3*	20	20	9.2*
Methionine, mg.	20*		66.7*	8*	5	5	20*	10*	20*	20*	20*	5.3*	10*	20*	4.6*
Norleucine, mg.			66.7*	20*	6*	6*						13.3*			9.2*
Norvaline, mg.			66.7*	20*								13.3*			9.2*
Phenylalanine, mg.	20*		66.7*	12*	8*	8*	20*		10*			8*	10*	20*	23*

TABLE 9—*Continued*

CONSTITUENT	1947													1948	
	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
Proline, mg.	20		66.7	5	7	7	20		10*	10*	10*	3.3		**10**	4.6
Serine, mg.	7.5*		66.7*	16*	12*	12*	20*		20*			10.7*		20*	9.2*
Threonine, mg.	20*		66.7*	90*	9*	**9***	20*		20*			60*	20*	**20***	9.2*
Tryptophan, mg.	20	5	66.7*	2	10	10	20*	†	10*	10*	10*	1.3	4	**10**	4.6*
Tyrosine, mg.	20*		66.7	6	6.5	6.5	20*	10	10			4	4	**10**	9.2
Valine, mg.	20*		66.7*	30*	12*	12*	20*		20*			20*	20*	**20***	34*
Casein (acid hydrolyzate), gm.		0.5[v]						0.5[s]		0.5[r]	0.5[s]				
Organism	F	F	B	M	M	F	F	F	B′	B′	B′	M	A	A-C-D F-Fer M	A

CONSTITUENT	1948											
	81	82	83	84	85	86	87	88	89	90	91	92
Glucose, gm.	2	1	1	2	4	1	2	2	1	1	1	2
$(NH_4)_2SO_4$, gm.	0.3			0.6		0.3				0.3		
NaAc, gm.	2.0	0.6	0.6	1.2	2.4	0.6	1.2	0.6	0.6[t]	0.6	0.6	1.2
NH_4Cl, gm.					1.2		0.6	0.3				0.6
KH_2PO_4, gm.	0.25	0.05	0.05	0.05	0.1	0.1	0.1	0.1	0.05	0.1	0.05	0.05
K_2HPO_4, gm.	0.25	0.05	0.05	0.05	0.1	0.1	0.1	0.1	0.05	0.1	0.05	0.05
$MgSO_4 \cdot 7H_2O$, gm.	0.08	0.02	0.02	0.02	0.04	0.02	0.02	0.02	0.02	0.02	0.02	0.02
NaCl, gm.	0.004	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001
$FeSO_4 \cdot 7H_2O$, gm.	0.004	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001
$MnSO_4 \cdot 4H_2O$, gm	0.016	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Inositol, γ						250		250		250		
Choline chloride, γ						250		250		250		
Thiamine·HCl, γ	100	20		100	200	10	100	50	100	10	20	100
Pyridoxine·HCl, γ	100	40[d]	20	160	320	30	160	160	20	30	200	160

Ca-Pantothenate, γ	100	20	20	200	400	10	200	20	20	10	20	200
p-Aminobenzoic acid, γ	5	4		0.01	0.02	30	0.1	0.1	20	30	4	0.01
Riboflavin, γ	100	20	20	200	400	10	200	20	20	10	20	200
Nicotinic acid, γ	100	20	20	200	400	10	200	20	40	10	20	200
Biotin, γ	0.5	0.02	0.01	0.5	1.0	0.02	0.5	0.1	0.5	0.02	0.02	0.5
Folic acid, γ	0.5	0.2	10	200	0.4	1	0.4	0.4	S	1	0.2	0.2
Adenine sulfate, mg	1.0	1.0	2.0	1.2	2.4	1.0	1.2	1.0	5	1	1.0	1.2
Guanine·HCl, mg	1.0	1.0		1.2	2.4	1.0	1.2	1.0	5	1	1.0	1.2
Uracil, mg	1.0	1.0		1.2	2.4	1.0	1.2	1.0	5	1	1.0	1.2
Xanthine, mg	1.0					1.0						
Alanine, mg	20*	20*	20*	200*	400*	20*	100*	20*	40*	20*	20*	100*
Aspartic acid, mg		20*		†	†	40	200*	40*	40	40	20*	
Asparagine, mg	40		20*	40	80							40
Arginine·HCl, mg	10	20	20	8	16	10	20	10	20	10	20	40
Cystine, mg	10	20	20	12	24	10	20	10	20	10	20	12
Glutamic Acid, mg	50	20*	20*	15	30	100	80*	40*	40	100	20*	15
Glycine, mg	10	20	20	10	20	10	10	10	20	10	20	10
Histidine·HCl·H_2O, mg	10	20	20	10	4	10	10	10	20	10	20	2
Hydroxyproline, mg		20	20		20				20	10	20	
Isoleucine, mg	20*	20*	20*	15*	30*	20*	20*	20*	20*	10*	20*	15*
Leucine, mg	10	20*	20*	7.5	15	10	20*	20*	20	10	20*	7.5
Lysine·HCl, mg	20	10	20*	16*	32*	10	20	10	20*	10	10	16
Methionine, mg	20*	20*	20*	4*	8*	20*	10*	20*	20*	20*	20*	4*
Norleucine, mg		20*	20*	10*	20*	20*		20*	20*	20*	20*	
Norvaline, mg				10*	20*	20*				20*		
Phenylalanine, mg	20*	20*	20*	6*	12*	20*	10*	20*	20*	20*	20*	6*
Proline, mg	10	20	20	2.5	5		10	10	20	10	20	2.5
Serine, mg	10	20*	20*	8*	16*	20*	†	†	20*	20*	†	8*
Threonine, mg	20*	20*	20*	45*	90*	20*	†	†	20*	20*	†	45*
Tryptophan, mg	10*	40*	20	1.0	2	10	5	10	20	10	40*	1.0
Tyrosine, mg	10	20	20	3.0	6	10	10	10	20	10	20	3.0
Valine, mg	20*	20*	20*	15*	30*	20*	20*	20*	20*	20*	20*	15*
Casein (acid hydrolyzate), gm.												
Organism	F	F	F	M	M	A	M	M	F	C	D	M

TABLE 9—*Concluded*

* DL—form.

Italics—denotes amino acid required by organism.

Bold face or †—denotes amino acid determined.

A = *L. arabinosus* 17-5; B = Lactic acid bacteria, 23 strains; B′ = Lactic acid bacteria, 8 strains. In some experiments sodium citrate substituted for sodium acetate and different combinations of salts, amino acids and buffers used; C = *L. casei*; F = *L. delbrueckii* LD-5; G = Glutamine; M = *L. mesenteroides* P-60; M′ = *L. mesenteroides*, No. 535; P = *L. pentosus*; S = Supplement; T = E. typhosa.

[a] = Glutamine, 2.5 mg.; [b] = Glutamine, required; [c] = Plus xylose, 0.1 gm.; [d] = Pyridoxamine·2HCl; [e] = Plus succinic acid, 1 gm.; [f] = Plus sodium citrate, 2 gm.; [g] = Glutamine determined; [h] = Plus pyridoxamine·2HCl, 120 gamma; [j] = Pyridoxamine·2HCl, 40 gamma; [k] = Plus pyridoxal, 2 gamma; [m] = Plus pyridoxamine·2HCl, 10 gamma and pyridoxal, 10 gamma; [n] = Plus glutamine, 0.5 mg.; [p] = Cysteine·HCl; [q] = Free base; [r] = H_2O_2 oxidized; [s] = Peptone, H_2O_2 oxidized; [t] = Plus succinic acid, 1 gm.; [u] = Pyridoxal; [v] = Plus casein tryptic digest, 0.06 gm.; [x] = Sodium citrate; [y] = Sodium acetate·$3H_2O$; [z] = Pyridoxamine·2HCl, 20 gamma.

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of Papers, Amer. Chem. Soc., New York, Sept. 11–15, 1944, p. 28) found better standard curves with glutamine than glutamic acid. *L. arabinosus* 17-5 specific for L-glutamic acid. Pyrrolidine carboxylic acid inactive. Determined L-glutamic acid in foods.

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fibroin, edestin, horse hemoglobin, and horse carboxyhemoglobin. Glutamine also determined with *L. arabinosus* 17-5. Aspartic acid determined with *L. Mesenteroides* P-60 in the same proteins. Glutamine more active than glutamic acid for *L. arabinosus* 17-5, while aspartic acid more active than asparagine for *L. mesenteroides* P-60.

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87. MEINKE, W. W. AND B. R. HOLLAND. *J. Biol. Chem.* 173: 535, 1948. Studied threonine-serine antagonisms with *L. mesenteroides* P-60, on their Medium A. No assays run.

88. MEINKE, W. W. AND B. R. HOLLAND (87). Medium B for use with *L. mesenteroides* P-60.

89. MEINKE, W. W. AND B. R. HOLLAND (87). Medium II of Baumgarten *et al.* (*Cereal Chem.* 23: 135, 1946) with xanthine omitted used with *S. faecalis* R.

90. MEINKE, W. W. AND B. R. HOLLAND (87). Medium of McMahan and Snell (*J. Biol. Chem.* 152: 83, 1944) with xanthine omitted used with *L. casei*.

91. MEINKE, W. W. AND B. R. HOLLAND (87). Medium of Stokes *et al.* (*J. Biol. Chem.* 160: 35, 1945) substituting pyridoxine HCl for pyridoxamine. Used with *L. delbrueckii*.

92. HELLER, G. L. AND E. R. KIRCH. *J. Am. Pharm. Assoc.* 36: 345, 1947. *L. mesenteroides* P-60 for determination of proline, isoleucine and cystine. Medium similar to Medium D of Dunn *et al.* (*J. Biol. Chem.* 156: 703, 1944) except that norleucine, norvaline and hydroxyproline were omitted. Arginine was increased fivefold. Alanine level was cut in half. Acid production increased approximately 3 ml. at high amino acid levels.

these amino acids was required for growth of the organism on an enriched basal medium of the composition shown in table 7. Confirmatory evidence may be needed, therefore, before the reliability of the values for these amino acids in natural products can be accepted without reservation.

In 1945 and 1946 Stokes *et al.* (51, 166, 167) described procedures for the determination of 9 amino acids with a single basal medium (No. 32, table 9) using one organism (*S. fecalis* R). As shown in table 12, later workers have followed this trend although there has been a tendency to employ *L. mesenteroides* P-60 in conducting assays in this manner. From 5 to 15 amino acids were determined with this organism in each of five laboratories referred to in this table. The question still remains, however, whether or not all, or any large number of amino acids, can be determined as satisfactorily in this way as by the use of different basal media and organisms. The data shown in table 13 indicate the variations in the composition of the basal media (table 9) which have been employed for the determination of amino acids with *L. mesenteroides* P-60. It has not been determined, however,

TABLE 10. AMINO ACIDS WHICH HAVE BEEN DETERMINED WITH LACTIC ACID BACTERIA[1]

ORGANISM	ALANINE	ARGININE	ASPARTIC ACID	CYSTINE	GLUTAMIC ACID	GLYCINE	HISTIDINE	HYDROXY PROLINE	ISOLEUCINE	LEUCINE	LYSINE	METHIONINE	PHENYLALANINE	PROLINE	SERINE	THREONINE	TRYPTOPHAN	TYROSINE	VALINE
L. arabinosus 17-5.....				X	X				X	X		X	X			X	X	X	X
L. casei[2].....		X	X		X				X	X			X		X		X	X	X
S. faecalis R.....	X	X	X		X		X		X	X	X	X			X	X	X	X	X
L. fermenti 36.....							X					X				X			
L. mesenteroides P-60..	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X

X Unpublished experiments, Rockland, L. B. and M. S. Dunn.

[1] Data taken from table 9 and Dunn, M. S. and L. B. Rockland. *Advances in Protein Chemistry* 3: 295, 1947.

[2] Same as *L. delbrueckii* according to Rogosa (139) and Dunn (34).

whether or not assays with these different media would yield results of the same or markedly different precision and accuracy.

The experiment whose results are summarized in table 14 was undertaken in the writer's laboratory in order to compare different organisms and basal media in assays of amino acid test mixtures and casein. Although the recoveries of threonine from 4 amino acid test mixtures containing from 0.5 to 4.0 per cent of threonine ranged from about 94 to 104 per cent, the values found for the percentage of threonine in casein varied only from 4.24 to 4.36 per cent. It seems probable that the agreement would be somewhat better if this experiment were to be performed according to the improved techniques now available.

The data from another experiment of interest in this connection are summarized in table 15. A test mixture containing alanine and serine in addition to the 16 amino acids listed in the table was assayed in each of six laboratories by the microbiological procedure in vogue. It may be noted that alanine and serine were not determined, that the values reported for cystine, glycine and proline are not satisfactory and that

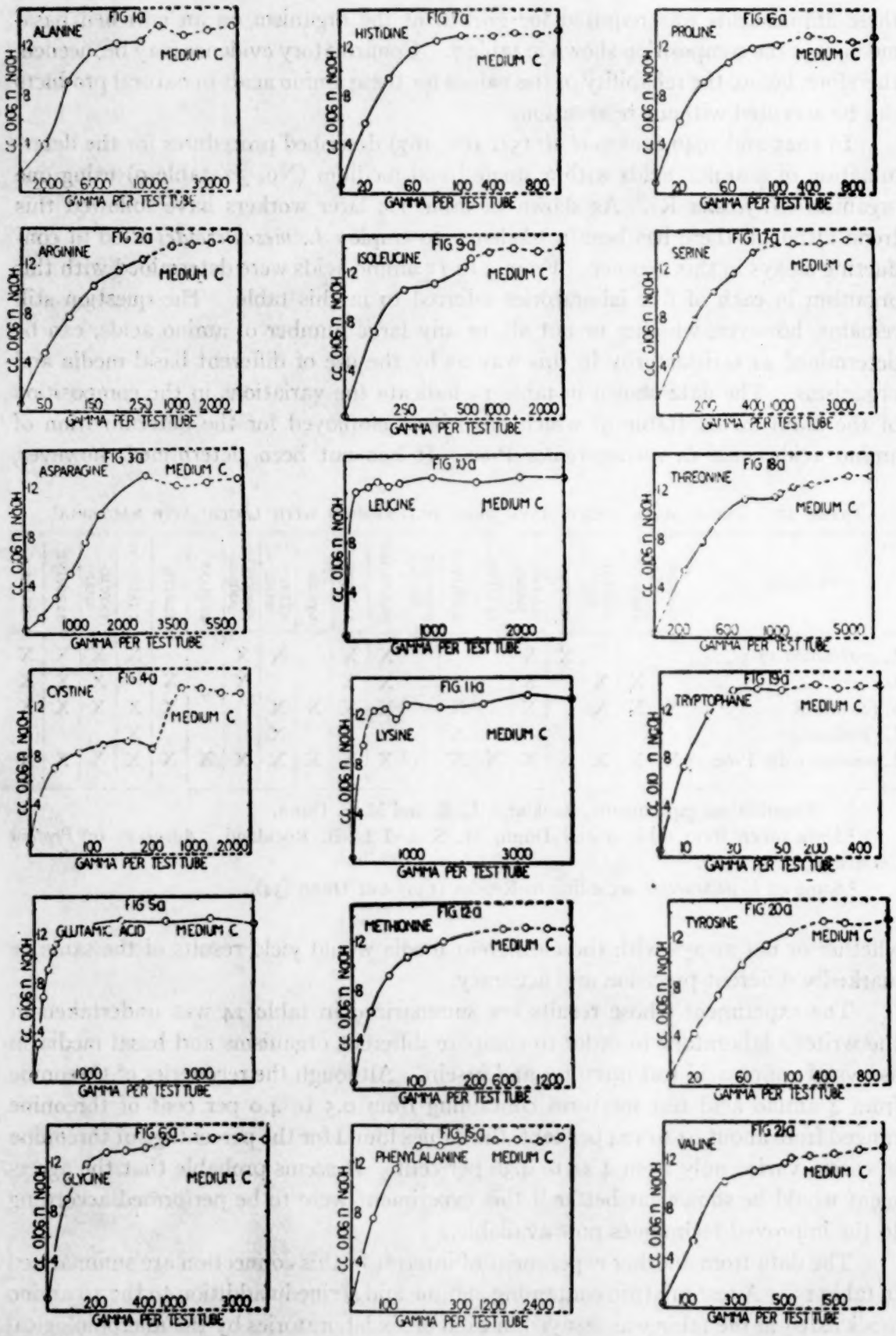

Fig. 3. STANDARD CURVES for 18 amino acids essential for growth of L. mesenteroides P-60 (DUNN, M. S., S. SHANKMAN, M. N. CAMIEN, W. FRANKL AND L. B. ROCKLAND. *J. Biol. Chem.* 156: 703, 1944).

acceptable values were obtained only in about half of the laboratories for arginine, histidine, lysine, threonine and tryptophan. There is a possibility, however, that some of the recoveries may prove to be better than those recorded in the table since there has been no opportunity to make certain that all of the values have been calcu-

TABLE 11. AMINO ACIDS DETERMINED IN NATURAL PRODUCTS WITH *L. mesenteroides* P-60

AMINO ACID	NATURAL PRODUCT	REF. NO.
Alanine	Corn steep liquor	1
Arginine	Corn steep liquor, enzymes	1, 2
Aspartic acid	Food proteins, viruses, bacterial toxins, enzymes	1–5, 16, 17
Cystine	Corn steep liquor, urine, enzymes	1, 2, 5
Glutamic acid	Enzymes	2
Glycine	Casein, silk fibroin, viruses, bacterial toxins, enzymes	2, 6, 16, 17
Histidine	Food proteins, viruses, bacterial toxins, enzymes	1, 2, 4, 7, 8, 9, 10 11, 17
Isoleucine	Food proteins, enzymes	2, 4
Leucine	Enzymes	2
Lysine	Corn steep liquor, food proteins, urine, enzymes, viruses, bacterial toxins	1, 3, 4, 5, 7–9, 12, 13, 16, 17
Methionine	Corn steep liquor, wheat, enzymes	1–3, 8
Phenylalanine	Corn steep liquor, enzymes	1–3, 8, 14
Proline	Corn steep liquor, enzymes, viruses, bacterial toxins	1–5, 15–17
Serine	Corn steep liquor, enzymes, urine	1, 2, 5
Threonine	No assays reported	—
Tryptophan	Food proteins	Author, unpubl.
Tyrosine	Food proteins, enzymes, urine	2, 4, 5
Valine	Enzymes	2

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lated on the same basis. The most striking conclusions to be drawn from this experiment are that the recoveries averaged 101 per cent for 66 of the 79 amino acid determinations and that about 80 per cent of the 66 values were within 100 ± 3 per cent of the correct figures. These findings strongly support the belief that many, if not all, amino acids can be determined with sufficient accuracy for many purposes

using different organisms and different basal media. It is recognized, however, that there is urgent need for improvement in basal media and methods applicable to the determination of certain amino acids in the assay of purified proteins, physiological fluids and other materials of theoretical and practical importance.

The composition of basal media employed currently for amino acid assays has been determined more or less arbitrarily. The concentration of carbohydrate,

TABLE 12. LACTIC ACID BACTERIA USED TO DETERMINE AMINO ACIDS IN SIX LABORATORIES

AMINO ACID	BARTON-WRIGHT	CARDINALL & HEDRICK	DUNN *et al.*	HENDERSON & SNELL	STOKES *et al.*	VELICK & RONZONI
Alanine		M				
Arginine	F	M	C	F or D	F	M
Aspartic acid	M	M	M	M	D	M
Cystine	A	M	A or M			M or A
Glutamic acid		A	A	A		M
Glycine			M			M
Histidine	M	M	M	F or M	F	M
Isoleucine	A	A	A	M	F	M or A
Leucine	A	A	A	A	F	M or A
Lysine	M	M	M	M	F	M
Methionine	M	M	A or FER.	F	F	M or FER.
Phenylalanine	M	M	M	A	D	M
Proline	M	M	P*	M		M
Serine		M			D	M
Threonine	F	F	FER.	F	F	FER.
Tryptophan	A		A	A	F	
Tyrosine			C	M	D	M
Valine	A	A	A	A	F	M or A

Notations: A, *L. arabinosus* 17-5; C, *L. casei*; D, *L. delbrueckii*; F, *S. faecalis* R; FER., *L. fermenti* 36; M, *L. mesenteroides* P-60; P, *L. breirs*.

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ammonium chloride, amino acid, phosphate buffer and inorganic salts in Uschinsky's original (1893) medium has been changed somewhat and numerous other nutrients have been added. Types and proportions of phosphate and other inorganic salts, essentially the same as those proposed by Speakman (163) in 1923, have been adopted by most workers. Following the isolation of thiamine (63) in 1926, vitamins were introduced into basal media as soon as they became available in crystalline form.

Basal media containing as many as 19 amino acids have been employed since 1922 to grow microorganisms and since 1936 to cultivate lactic acid bacteria. Although

TABLE 13. RANGE OF CONCENTRATIONS OF NUTRIENTS IN BASAL MEDIA USED TO DETERMINE AMINO ACIDS WITH LEUCONOSTOC MESENTEROIDES P-60

	%		%		%
Glucose	1–4	Biotin	0.1–1.0	DL-Isoleucine	15–40
$(NH_4)_2SO_4$	0–0.6	Folic acid	0.1–1.0	L-Leucine	7.5–20
NaAc	0.6–2.4	Pyridoxamine·HCl	0–120	DL-Leucine	20–20
NH_4Cl	0–1.2	Adenine sulfate·$2H_2O$	1.0–2.4	L-Lysine·HCl	10–32
KH_2PO_4	0.05–1.6	Guanine·HCl·$2H_2O$	1.0–2.4	DL-Lysine·HCl	10–32
K_2HPO_4	0.05–1.6	Uracil	1.0–2.4	DL-Methionine	4–20
$MgSO_4 \cdot 7H_2O$	0.02–0.04	Xanthine	0–2.0	DL-Norleucine	0–40
NaCl	0.001–0.5	DL-Alanine	20–400	DL-Norvaline	0–40
$FeSO_4 \cdot 7H_2O$	0.001–0.002	L-Aspartic acid	40–80	DL-Phenylalanine	6–40
$MnSO_4 \cdot 4H_2O$	0.001–0.002	DL-Aspartic acid	40–200	L-Proline	2.5–20
Inositol	0–250	L-Asparagine	20–80	DL-Proline	20–20
Choline chloride	0–250	L-Arginine·HCl	8–60	DL-Serine	8–40
Thiamine chloride	50–200	L-Cysteine·HCl	16–16	DL-Threonine	20–90
Pyridoxine·HCl	100–1000	L-Cystine	10–24	L-Tryptophan	1–20
Ca pantothenate	20–400	L-Glutamic acid	15–200	DL-Tryptophan	10–10
P-Aminobenzoic acid	0.01–50	Glycine	10–20	L-Tyrosine	3–10
Riboflavin	20–400	L-Histidine·HCl·H_2O	2–20	DL-Tyrosine	10–10
Nicotinic acid	20–400	L-Hydroxyproline	0–20	DL-Valine	15–40

TABLE 14. THREONINE IN FOUR AMINO ACID TEST MIXTURES[1] AND CASEIN[2] DETERMINED WITH THREE LACTIC ACID BACTERIA AND FOUR BASAL MEDIA

SAMPLE	S. FAECALIS R		L. ARABINOSUS	L. FERMENTI
	Stokes	Greenhut	Hier	Dunn
Test mixt., 0.5% threonine	93.5	104	97.5	97.7
Test mixt., 1.0% threonine	94.5	101	97.5	97.9
Test mixt., 2.0% threonine	93.4	104	98.5	96.0
Test mixt., 4.0% threonine	93.1	104	100	94.8
Casein, moisture-ash free	4.25	4.36	4.36	4.24

[1] Values given as per cent recovery. [2] Per cent threonine.
[3] Data in table obtained in writer's laboratory.

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reasonably satisfactory assay procedures for the determination of amino acids have been elaborated, it is not to be assumed that any available method approaches perfection.

A study of basal media to be used in the quantitative determination of amino acids in protein materials was initiated by Dunn and co-workers (35) in 1944. Although it was desired that this investigation should be systematic and complete, rather than empirical and limited, it was recognized that a prohibitive length of time would be required to determine the environmental conditions and the types, proportions and levels of nutrients which are optimal for the growth of lactic acid bacteria. In this work the minimum quantity of each amino acid required to give

TABLE 15. DATA FOR RECOVERY OF AMINO ACIDS FROM TEST MIXTURE DETERMINED BY MICROBIOLOGICAL ASSAY IN SIX LABORATORIES

AMINO ACID	% RECOVERY BY LABORATORY						AMINO ACID	% RECOVERY BY LABORATORY					
	1	2	3	4	5	6		1	2	3	4	5	6
Arginine	98	*113*	94	107	*84*	103	Lysine	101	103	104	*87*	*78*	104
Aspartic acid	99	99		98		102	Methionine	99	109	101	100	99	102
Cystine		*76*				*127*	Phenylalanine	103	100	99	103	101	100
Glutamic acid	101	101		100	105	100	Proline		*117*		101		
Glycine	104					107	Threonine	100	100	101	*111*	*114*	94
Histidine	99	104	102	*114*	*80*	108	Tryptophan		109	*126*	100	*125*	102
Isoleucine	99	102	104	99	99	105	Tyrosine		99	100	101	109	100
Leucine	100	97	99	98	*90*	101	Valine	101	103	98	101	99	103
Range[1]								98–104	97–109	94–104	98–107	99–109	94–108
Average[1]								100	102	100	101	102	102

[1] Italic values omitted in determining ranges and calculating averages.

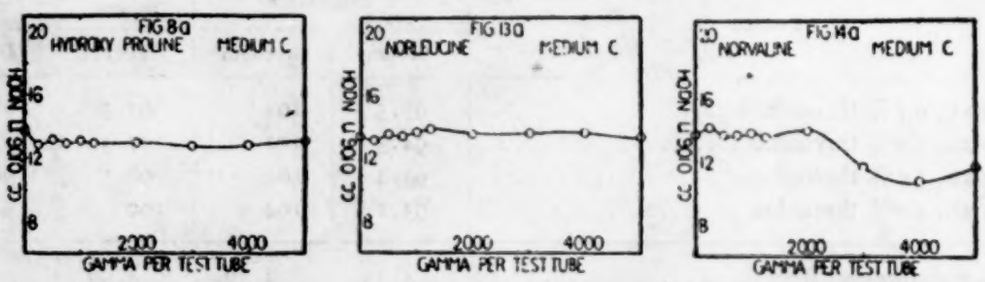

Fig. 4. STANDARD CURVES for non-essential amino acids, hydroxyproline, norleucine and norvaline (DUNN, M. S., S. SHANKMAN, M. N. CAMIEN, W. FRANKL AND L. B. ROCKLAND. *J. Biol. Chem.* 156: 703, 1944).

the maximum titration and the minimum level of the amino acids as a whole at which the slope of the standard curve for a particular amino acid was maximum, were determined using a basal medium of arbitrary composition. Then a new basal medium was prepared of the same composition as the original except that each essential amino acid was introduced at the minimum level found to be optimum. If the minimum amounts of an amino acid required to give maximum titrations did not differ significantly in the two experiments, it was assumed that the indicated proportion of the amino acid was optimum. If necessary, this experimental procedure was repeated

until a constant minimum level of each amino acid resulted. Finally, the composition of the near-optimum basal medium was determined from these data.

The amino acid requirements of *Leuconostoc mesenteroides* P-60 (35), *Lactobacillus fermenti* 36 (26) and about 20 other lactic acid bacteria (34), as well as some of the vitamin (148) and carbohydrate (17) requirements of these organisms, have been determined in this manner. It has been possible to develop assay procedures for the determination of glutamic acid (25), glycine (149), histidine (30, 31, 33), leucine (16), lysine (29), methionine (27), phenylalanine (32), threonine (28) and some other amino acids for which methods have not been reported. A microbiological procedure for the determination of proline with *Lactobacillus brevis* is to be described in a forthcoming paper.[8] It is of interest in this connection that Boyd *et al.* (8, 9) have recently determined the amino acid requirements of *Clostridium per-*

TABLE 16. ORGANISMS OTHER THAN LACTIC ACID BACTERIA USED TO DETERMINE AMINO ACIDS

AMINO ACID	ORGANISM	REF. NO.	AMINO ACID	ORGANISM	REF. NO.
Amino acids (thirteen)	*Cl. perfringens* BP6K	(1)	Methionine	*E. coli* 532-171	(3, 9)
Arginine	*E. coli* 1577-28	(2, 3)	Phenylalanine	*E. coli* mutants	(10)
Histidine	*Tetrahymena geleii* H	(4)	Tryptophan	*E. coli* mutant	(3)
Leucine	*Neurospora crassa* 33757	(5–7)	Tryptophan	*Tetrahymena geleii* H	(11)
Leucine	*E. coli* 679-680	(8)	Tyrosine	*E. coli* 58-5030	(10)

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fringens (Welchii) BP6K and have reported a procedure for the determination of 13 amino acids with this organism. A possible disadvantage may be that this strain of Clostridium is potentially a toxin former. A microbiological assay procedure for alonine has been reported by Sauberlich and Baumann.[9]

Since it has been established that microbiological data for amino acids vary in precision and accuracy depending upon the amino acid determined, the protein or other amino acid mixture assayed, the lactobacillus strain used as the test organism, and the control exercised over the environmental conditions it may be that basal media whose composition has been determined on logical premises may yield analytical results of higher quality than those obtained under other conditions. On the other hand it may very well be true that acceptable data for some, if not all, purposes

[8] See table 12 for reference to proline paper.

[9] Sauberlich, H. E. and C. E. Baumann. *J. Biol. Chem.* 177: 545, 1949.

TABLE 17. TOTAL AND 'FREE' AMINO ACIDS IN NORMAL URINE

AMINO ACID	TOTAL		FREE		AMINO ACID	TOTAL		FREE	
	(1)	(2)	(3)	(2)		(1)	(2)	(3)	(2)
Arginine	30	24	9.5	21	Leucine	29	21	7.2	9.6
Aspartic acid	175	165		1.3	Lysine	84	73	47	34
Cystine			108(4)	88	Methionine	14	8.6	4.4	7.8
Glutamic acid	347	351		36	Phenylalanine	29	23	14	16
Glycine	714				Threonine	49	54	21	24
Histidine	170	203	163	188	Tryptophan*			18(4), 20(5)	25
Isoleucine	19	20	14	5.9	Valine	27	20	7.4	4.5

* Albanese and Frankston (6) found 281 mg. and Berg and Rohse (7) found 164 and 26 mg. of tryptophan in normal urine by colorimetric methods.

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TABLE 18. PERCENTAGE OF AMINO ACIDS IN ORGANS OF MAMMALS AND BIRDS[1]

AMINO ACID	BLOOD	CARCASS	HEART	KIDNEY	LIVER	MUSCLE	SKIN
Arginine							
mammal	3.6–4.4	4.8–5.6	5.2–6.4	5.2–6.3	5.4–6.5	5.4–6.3	7.6–8.8
bird	4.7–5.1	5.3–5.9	5.3–6.4	5.6–6.7	5.1–6.7	5.7–6.1	5.3–7.0
Aspartic acid							
mammal	11–14	7.8–10	10–11	9.7–11	10–12	9.7–11	6.5–7.5
bird	9.8–11	7.6–10	10–11	9.5–11	10–11	9.7–11	6.3–9.3
Glutamic acid							
mammal	7.6–12	11–14	9.3–15	11–12	14–15	16–18	11–14
bird	9.5–10	11–12	13–16	11–12	14–16	16–18	10–11
Glycine							
mammal	3.5–6.9	6.9–10	5.5–9.4	5.4–7.3	5.2–6.5	4.3–7.1	12–15
bird	2.8–4.4	7.7–13	5.0–5.8	4.5–6.0	5.2–7.8	4.6–6.7	8.0–13
Histidine							
mammal	5.7–7.5	1.7–2.0	2.5–3.6	2.3–4.2	2.5–2.9	1.9–2.4	1.1–1.4
bird	5.4–5.9	1.7–2.0	2.5–2.9	2.7–3.6	2.7–3.0	2.2–2.3	0.6–1.2
Isoleucine							
mammal	1.7–3.6	3.4–4.4	4.2–5.2	4.0–4.8	5.0–5.7	4.3–5.3	2.2–2.6
bird	4.4–4.9	3.7–4.5	4.9–5.4	4.6–5.4	5.2–6.2	4.6–5.2	3.4–6.2

[1] *Mammals:* Cat, dog, mouse, rabbit and rat. *Birds:* Chicken, duck and turkey. Values corrected to 16% nitrogen. Data from Dunn *et al.*, *U. Calif. Publ. Physiol.* 8: 293, 1949.

can be acquired using basal media varying widely in the types and proportions of their nutrient substances.

The following questions are examples of those which must be answered before one can determine the quality of the amino acid data obtained for proteins, foods and physiological fluids. What stimulatory and inhibitory influences on the growth of lactic acid bacteria are exerted by various derivatives, analogs, homologs and antipodes of the amino acids in blood, urine and protein hydrolyzates? What is the effect of peptides (including strepogenin) on the growth of these organisms? How can cystine, tryptophan and some other labile amino acids be liberated from proteins without undergoing the destruction resulting from acid or base hydrolysis? What reactions occur between carbohydrates, vitamins, amino acids and other nutrients during autoclaving of basal media and to what extent do they lower the precision

TABLE 19. PERCENTAGE OF AMINO ACIDS IN ORGANS OF MAMMALS AND BIRDS[1]

AMINO ACID	BLOOD	CARCASS	HEART	KIDNEY	LIVER	MUSCLE	SKIN
Leucine							
mammal	11–13	6.2–7.1	7.8–13	7.6–8.8	8.9–9.9	7.0–8.1	4.2–5.6
bird	10–11	6.7–7.8	8.2–9.0	8.3–9.4	9.2–10	7.3–7.8	6.6–7.9
Lysine							
mammal	9.2–11	5.5–9.0	7.6–8.9	6.7–7.7	6.9–8.2	7.7–9.0	3.7–5.2
bird	8.3–10	6.4–8.9	7.0–8.2	7.6–8.3	7.3–8.0	8.3–8.8	2.4–5.0
Methionine							
mammal	0.8–1.8	2.0–2.5	1.6–2.5	2.0–2.5	2.0–2.4	2.3–2.7	0.9–1.2
bird	1.2–1.6	1.8–2.5	2.2–2.9	2.0–2.2	2.0–2.4	2.3–2.7	0.8–1.1
Phenylalanine							
mammal	6.6–8.0	3.1–3.7	4.2–4.9	3.5–4.8	4.8–5.5	3.5–4.0	2.3–3.0
bird	5.8–6.7	3.0–3.6	4.1–4.8	4.7–5.3	4.8–5.1	3.7–3.9	3.8–4.6
Threonine							
mammal	4.5–6.3	3.6–4.0	4.4–4.8	4.0–4.8	4.3–5.0	3.9–5.0	3.0–4.2
bird	4.6–5.3	2.7–4.0	3.9–4.9	3.9–5.0	3.6–4.7	3.5–4.5	2.8–5.1
Valine							
mammal	7.2–8.8	4.2–5.0	4.7–5.9	5.4–6.4	6.1–6.6	4.5–5.1	3.3–4.2
bird	7.0–8.0	4.1–4.8	5.6–6.2	5.9–6.5	6.4–6.7	4.7–4.9	5.8–7.9

[1] *Mammals:* Cat, dog, mouse, rabbit and rat. *Birds:* Chicken, duck and turkey. Values corrected to 16% nitrogen. Data from Dunn *et al.*, *U. Calif. Publ. Physiol.* 8: 293, 1949.

and accuracy of amino acid assay data? How can any difficulties of this kind be circumvented? Can amino acid assays be made with as much, or greater, convenience and dependability with Tetrahymena, Clostridium, mutants of *Neurospora crassa* or *E. coli* and other organisms of the types shown in table 16?

Despite any shortcomings inherent in the microbiological procedures available today for the determination of amino acids, it is evident that these methods are being used increasingly and that they have almost unlimited application to the determination of amino acids in many, if not all, types of biological materials. Examples are given in table 17 of the amino acid data for normal urine obtained in 3 laboratories and values for amino acids in different organs and tissues of a number

of laboratory or domestic mammals and birds are shown in tables 18 and 19. It would not have been feasible, previously, to undertake studies of such scope.

It may be said, finally, that the developments in the microbiological assay of amino acids which have been discussed may serve to illustrate anew the statement that *"The greatest advances come, not from the refinement of old methods, but by the discovery of new tools."*

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EXOPHTHALMOS

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FOLLOWING THE DESCRIPTIONS of exophthalmic goiter by Plummer (97), Graves (41), Basedow (9) and others, many workers have studied the production of exophthalmos in lower animals. Their results have made clear the paths from cerebral neurones to the smooth muscle and vessels of the orbit. They have laid sound foundations for the treatment of exophthalmos in lower animals. Though their results cannot be applied directly to cases of exophthalmos in man owing to anatomical differences in the human orbit, the production of exophthalmos by an anterior pituitary principle is of great interest to physicians and surgeons.

EXPERIMENTAL EXOPHTHALMOS IN LOWER ANIMALS

Perhaps the first worker to throw light on the mechanism of exophthalmos was Claude Bernard (11). He showed that if the cervical sympathetic nerve trunk in the dog was cut, the eyeball receded and the upper lid drooped. If the peripheral (cephalic) end of the cut nerve was stimulated electrically, these effects were reversed. In later work Bernard (12) showed the spinal segments from which fibers pass in the cervical sympathetic trunk to the pupil and to the vessels of the head, but he made no report on the fibers which altered the position of the globe in relation to the orbit.

In 1858 Müller (85) published a 'preliminary communication' on the smooth muscle of the orbit in men and animals. The first two paragraphs of it may be translated as follows:

> In man the inferior orbital fissure is filled with a greyish red mass. This consists of bundles of smooth muscle fibers which are usually equipped with elastic tendons. In mammals there is a strongly developed muscular membrane connected with elastic laminae which is its more strongly developed analogue and which consists also of smooth muscle fibers (musculus orbitalis or orbital membrane of the author).

After a paragraph on the nictitating membrane the communication concludes:

> The orbital muscle is supplied with nerve bundles which consist almost entirely of fine or non-medullated (sympathetic) fibers. The nerves can be traced anatomically in part to the sphenopalatine ganglion. The orbital muscle produces, by its contraction, the protrusion of the globe which has been observed in animals during the stimulation of the cervical sympathetic nerve. The same muscle acts as antagonist to the muscles which move the globe backwards in its cavity (M. retractor, orbicularis palpebrarum).

Müller's untimely death shortly afterwards prevented him from writing a fuller account of this orbital muscle or membrane which has been known since as 'Müller's orbital muscle' and is to be distinguished from the smooth-fibered part of the levator palpebrae superioris or superior tarsal muscle (100) to which the name of Müller's palpebral muscle is often given. Excepting the figure from Harling's article (44),

no illustrations of this muscle were available when the present writer was studying differences between the muscles of lower mammalian and human orbits. Photomicrographs of the muscle in the dog and cat are given by Brunton (18).

Harling (44) studied the orbital muscle in the sheep, dog, and man and illustrated the muscle in sheep. He could not believe that the muscle in the infra-orbital fissure of man is able to draw the eye forward and most later writers (134) agree with this conclusion. In the dog and other lower mammals, however, the orbital muscle is a hollow cone with its apex near the optic foramen and sphenoidal canal and its base attached to the anterior orbital ring. Prévost and Jolyet (103) showed that the orbital muscle contracted when the cervical sympathetic nerve trunk was stimulated.

MacCallum and Cornell (69) published a classical account of their investigations on exophthalmos which described both dissections of human and canine orbits and a number of recorded responses to stimulation of the cervical sympathetic nerve trunk. In experiments on the dog they removed the roof, external wall and much fatty tissue from the orbit. This enabled them to observe the contraction of Müller's orbital muscle. They recorded the movements of the eyeball on a moving drum by a lever. One end of this lever was fitted with a writing point and the other end lay on the dog's cornea. In this way they avoided the illusion of exophthalmos which can be produced by lid retraction. The contraction of the muscular cone could be seen during stimulation of the nerve and the movement of the globe was recorded on the drum by the writing point of the lever. When they replaced the eyeball by a balloon, stimulation of the cervical sympathetic nerve trunk increased the pressure in the balloon so that it advanced in the orbit, and thus showed that no muscular attachment was necessary to draw the eyeball forward. When the position of the balloon was kept constant, its internal pressure increased when the nerve was stimulated. In other experiments MacCallum and Cornell produced exophthalmos by obstructing the venous outflow from the orbit. They also showed in the cat that stimulation of the ventral root of the second thoracic nerve produced both exophthalmos and pupillary dilatation. Whitnall and Beattie (135) obtained results on cats similar to those of MacCallum and Cornell on dogs. Using spinal dogs under chloralose Code and Essex (24) demonstrated by motion photography the response of Müller's orbital muscle and of the eyeball to stimulation of the cervical sympathetic trunk. Stimulation of the vago-sympathetic trunk has failed to produce exophthalmos in apes and in human subjects (10, 60, 61, 69, 99). Indeed the mechanism of exophthalmos in man often remains doubtful. In the horse, sheep, dog, cat, rabbit and guinea-pig, however, the immediate cause of exophthalmos may be due either to Müller's orbital muscle or to changes in the blood supply of the orbit (19). Events in the body which influence these mechanisms will be considered next.

The path of nerve fibers beyond the superior cervical ganglion has been little studied since Müller stated that some fibers supplying his orbital muscle could be traced to the spheno-palatine ganglion. (His article did not show whether this had been done in man or a lower mammal.) The writer sought for a synapse in the spheno-palatine ganglion by infusing eserinized Ringer's solution containing acetyl choline into the artery which supplied the ganglion in the isolated head of the dog. After a few experiments the work was interrupted by the Second World War but a

provisional conclusion was reached that no post-ganglionic fibers originated at this site. The work did not disprove a hypothesis that fibers might pass through the ganglion without being relayed. Recently an article by de Kleijn and Socin (28) has come to the notice of the writer. These workers studied the post-ganglionic nerves to the nictitating membrane, the pupil and the orbital vessels in the cat. By a series of nerve sections and stimulations they traced these nerve fibers away from the internal carotid artery laterally into the middle ear, forward alongside but not in the wall of the eustacian tube to the foramen rotundum, piercing the basiphenoid lateral to the vidian nerve and medial to the second root of the trigeminal nerve and then either with the first part of the trigeminal nerve to the pupil or with some other nerve to the nictitating membrane and the smooth retractor muscle of the upper lids. Further study of this difficult path would be rewarding. It seems possible that the fibers to Müller's orbital muscle travel by the same path as these for the retractor of the upper lid.

The possibility of a nervous pathway from the stellate ganglion along the vertebral artery to the orbit was suggested by observations of Leriche and Fontaine (67) on human subjects. They noticed dilatation of the pupil after the cervical sympathetic trunk had been cut. This effect, however, might have been produced either by a rise of blood pressure in the orbital cavity or by a rise in the adrenalin content of the blood. Each of these conditions may be produced under normal conditions by emotion. A recession of the globe when dogs under anesthesia are spinalized has been noticed by Code (23) but this recession may depend upon a fall of general body blood pressure. Brunton (19) recorded the effect produced on the eyeball by stimulation of the stellate ganglion before and after cutting the cervical sympathetic trunk in dogs. No response in the orbit was obtained after cutting the nerve trunk, though stimulation of the cut distal end of the trunk still produced exophthalmos. The conclusion was drawn that usually no path for production of exophthalmos originated in or passed through the stellate ganglion in the dog except that carried by the fibers of the cervical sympathetic trunk.

The nerve path to Müller's orbital muscle has been gradually traced, in certain animals at least, from the cervical nerve trunk to the anterior root of the second thoracic nerve (69) to the region of the fourth ventricle (36), to the hypothalamic region and almost certainly in the cat to the frontal lobe of the brain. Following the work of Karplus and Kreidl (56, 57, 58), Bard (6, 7) studied the effects caused in cats by loss of the cerebral hemispheres, the corpora striata and the anterior half of the hypothalamic region with the corresponding part of the thalamus. Among the motor activities which followed the operation was exophthalmos. Bard suggested that the exophthalmos was due to release of posterior hypothalamic and lower centers from control by the brain areas which had been removed. Further work (42, 49) has shown the existence of sympathetic and parasympathetic centers in the hypothalamic region of the cat and monkey for control of the heart rate, of arterial caliber, of respiration, of heat production and heat loss. The parasympathetic points are anterior to the true sympathetic points. Kennard (55) has traced autonomic paths still farther forward into the frontal lobe of the brain of the monkey where stimulation produces not only somatic motor responses but also such autonomic responses as salivation, lacrimation, pilomotor and vasomotor changes. The study of human

response to emotions and the general integration of the human nervous system has led some to expect this closest possible relation between the two efferent systems at the highest levels. It is probable that a point in the frontal lobe will be discovered which, when stimulated, will produce exophthalmos. Thus the production of exophthalmos by Müller's orbital muscle is almost completely explained.

The production of exophthalmos by changes in the orbital circulation was studied by MacCallum and Cornell (69), by Beattie and Whitnall (135) and by Brunton (19). Interference with the venous outflow or an increase of blood pressure in the isolated head of the dog will produce up to 2.0 mm. of exophthalmos. Acetyl choline will produce exophthalmos by dilation of the orbital vessels but there is no evidence that this occurs in normal animals. In lower mammals the orbicularis and retractor bulbi muscles usually prevent exophthalmos. The retractor muscle has rarely been found in man. One case has been described by Whitnall (133).

So far the influence of endocrine products has not been considered. From the time of Graves (41) and Basedow (9) a connection was suggested between the thyroid gland and the exophthalmos. Sub-total thyroidectomies in man frequently relieved the patients' symptoms, but in a significant proportion of cases exophthalmos persisted after other symptoms had been removed, suggesting that the exophthalmos was not due to excessive thyroid secretion. Nevertheless, other cases were reported in which thyroid medication produced exophthalmos (15) and sub-total thyroidectomies usually removed the patients' troubles. These facts encouraged a study of the results of thyroid administration to lower animals. Early results were complicated by unsatisfactory methods of extraction of thyroid principles and by the unintentional removal of the parathyroid glands from experimental animals. By feeding sheep's thyroid Cunningham (27) produced exophthalmos in rabbits. Edmunds (33, 34) obtained it in rabbits and monkeys; Baruch (8), in bitches; Klose and others (59), in a fox terrier. In 1910 Gley (40) made the important assertion that he had effected exophthalmos in a rabbit after thyroidectomy, a report which was politely ignored until surgeons had reported many cases of exophthalmos after thyroidectomies in human subjects. Using extracts of thyroid glands or Kendall's thyroxin, Kunde (63) obtained slight exophthalmos in normal rabbits, marked exophthalmos in young thyroidectomized rabbits but no exophthalmos in 3 dogs. Although the effect of thyroid substances was still uncertain, the immediate aim of research carried out in the laboratories of the Medical Faculty at Paris was the production and study in dogs of the 'Basedow type of exophthalmos' by means of combinations of sympathomimetic substances including thyroid extract or thyroxin. This type of exophthalmos was defined as "an exophthalmos which involves neither increased tension of the eyeball, orbito-palpebral edema or mydriasis" (52). Dogs reacted to thyroxin in doses of 1 mg/kg/day by loss of weight and tachycardia but showed no exophthalmos. Doses of 0.5 mg/kg of ephedrine were also without effect but a combination of these two in either order produced a rapid and intense exophthalmos (54). The administration of pilocarpine could prevent mydriasis and so produce the Basedow type. Photographs were published and a cinema film was made (53). Boivin (14) found reason for thinking that thyroxin itself, working with adrenalin, actually opposes mydriasis.

Sensitization to adrenalin by denervation has been proved in the case of many

structures which are innervated by sympathetic nerves. Injury to such nerves was at one time a suggested cause of exophthalmos. In 1909 Eppinger and others reported that sensitization of the iris of the dog to adrenalin could be produced by thyroid products. Such sensitization has been reported for the auricle of the guinea-pig heart (62) and for the dog heart (3). The action of adrenalin is extended in time by ephedrine (39); it is potentiated by guanidine (21), by pyrogallic acid (4) and by acetyl choline (106). Most of this potentiation was proved on the nictitating membrane but the potentiation by pyrogallol has been confirmed by the present writer in respect of exophthalmos in dogs. The fact that exophthalmos produced by adrenalin outlasts the accompanying rise of blood pressure suggested that adrenalin acted apart from the blood pressure but *1*) exophthalmos produced by nerve stimulation also outlasts the stimulation (69) and *2*) in the isolated head of the dog, if the perfusion pressure is kept constant after injections of adrenalin almost no exophthalmos occurs (19). The exact conditions for the sensitization of exophthalmos by thyroxin are not yet known.

In 1928, Chesney, Clawson and Webster (22) reported that rabbits fed on a diet rich in cabbage developed hyperplastic goiters. In a series of papers, Marine and his co-workers (73–77) reported that exophthalmos occurred in rabbits which were subjected to a cabbage diet; that it occurred more regularly in young male rabbits of Dutch strain and that it could be produced by the injection of 0.1 to 0.25 cc. of methyl cyanide daily for 3 to 8 weeks (77); and that the cyanide produced the exophthalmos after thyroidectomy (74) but failed to do so after removal of the superior cervical ganglion. They suggested in explanation, that the cyanide produced a tissue anoxemia which stimulated the hypothalamic centers. The center then produced impulses which stimulated the anterior pituitary body to produce thyrotropic hormone. This hormone was thought to stimulate a hypothalamic center for exophthalmos besides acting on the thyroid gland (74). It seems possible however, that the anoxemia in the hypothalamic region might stimulate the exophthalmic mechanism without involving the pituitary hormones. The question remains uncertain.

Aron (1) using guinea-pigs first demonstrated the effect of pituitary extract on the mammalian thyroid gland. His work was confirmed by Loeb and Bassett (68) and by many other workers. An admirable review of the work between 1899 and 1938 was published by Lambie (64). Injections of anterior pituitary thyrotropic hormone (APTH) produced exophthalmos as one of their effects and it was found that the hormone produced exophthalmos in rabbits and guinea-pigs even more definitely after thyroidectomy (35, 75, 95, 119). Paulson (95) claims that all his thyroidectomized guinea pigs showed exophthalmos when given APTH. After removal of the superior cervical ganglion, APTH still produced exophthalmos in rabbits (35) and guinea-pigs though cyanide does not act after removal of the ganglion (74).

The changes produced by APTH in the orbit of thyroidectomized guinea-pigs have been studied by Smelser (119). Of 26 pigs (from which the left superior cervical ganglion had also been removed) 23 showed exophthalmos after daily treatment with extracts of 250 mg. of acetone-dried beef anterior pituitary. The exophthalmos was

definite in 12 to 20 days. There was an average increased weight of 100 per cent in the fatty connective tissue of the orbit, of 40 per cent in the dorsal lacrimal gland and of 22 per cent in the extra-ocular muscles. Lymphocytes were present in some cases. Reproductions of photomicrographs were given (120). If Harder's gland were removed from the pig, the increase in weight of the remaining orbital tissues was still 40 per cent after APTH (123). The primary change was edematous infiltration and hypertrophy of orbital fat. The hypertrophy of orbital muscle was in part secondary to the changes in other tissues (124). These changes closely resembled those found in cases of human post-thyroidectomy exophthalmos (119).

Smelser (122) refers to the fact that exophthalmos due to cyanide can be distinguished from that due to APTH. The former type is preventable or removable by *1*) excision of the superior cervical ganglion or *2*) by administration of iodine or fruit juice. The latter type is unaltered.

In their work with methyl cyanide Marine and Rosen (75) noticed that along with the development of exophthalmos there were often signs of precocious sexual development, particularly after thyroidectomy. In 1936 they reported (76) that exophthalmos was most easily produced in young male rabbits which were most active sexually. Gonadectomy was performed on 38 young male and 23 young female rabbits. In none of these would cyanide produce exophthalmos and in 3 rabbits which had developed frank exophthalmos the exophthalmos receded after gonadectomy. Other investigations were made at the same time but no final conclusions were drawn. Smelser (124) found that if estradiol was injected into female guinea-pigs their extra-ocular muscles increased in weight.

Thus, in guinea-pigs at least, exophthalmos may be produced by the use of APTH after thyroidectomy, by gonadal substances after thyroidectomy and by administration of cyanides. In cats and dogs exophthalmos has been reported following the administration *a*) of thyroid extracts with ephedrine or *b*) of adrenal extracts with a sensitizing substance which has sometimes been thyroid extract.

Thus, apart from stimulation of Müller's orbital membrane through sympathetic nervous paths, adrenalin with thyroid extract or some other sensitizing agent, APTH, cyanides or alterations in the orbital circulation might produce exophthalmos in lower animals. Which of these is the normal method of production? Unfortunately the lower animals seldom show exophthalmos. Where cases of thyrotoxicosis are seen in horses, dogs and other animals veterinary surgeons could do valuable work by recording and publishing the results of their treatment.

EXOPHTHALMOS OF ENDOCRINE OR NERVOUS ORIGIN IN HUMAN SUBJECTS

The purpose of this section is to review investigations which have been made on those conditions of exophthalmos in man which appear to be related either to the products of endocrine glands, to the autonomic nerves, to weakness of the extrinsic muscles of the eye or to a combination of these conditions. No further reference will be made to exophthalmos associated with oxycephaly, cavernous sinus thrombosis, high intra-ocular pressure, rat bite fever or tumours of the orbit, nasopharynx or cranial cavity.

The terms 'exophthalmos' and 'proptosis' will be used interchangeably (though

Mann (72) has defined them separately) and without implying any theory of the mechanism which causes the eyeball to advance in the orbital cavity.

In human subjects exophthalmos has been associated with action of the thyroid gland since Graves (41) and Basedow (9) described the disease which bears their names. Enquiry was first directed to the immediate mechanism by which the eyeball was made to advance in the bony orbit. Experiments on lower animals failed to help in this matter since their orbital anatomy is different from that of man. Enquiry also sought to ascertain exactly the chain of stimuli which set in motion the immediate mechanism. This enquiry uncovered the fact that exophthalmos might persist and increase after removal of the thyroid gland and might sometimes even be relieved by administration of thyroid extract. Results of experiments on animals were available at this stage to suggest that this post-thyroidectomy exophthalmos depended on anterior hypophyseal thyrotropin (called APTH in this paper). These experiments do not disprove the existence of exophthalmos due to thyroxin, but no exact explanation of thyrotoxic exophthalmos has so far received universal assent.

In order to produce exophthalmos a change must occur either in the tension of some orbital muscle or in orbital fluid pressures. This change constitutes the immediate mechanism (proximate cause) of exophthalmos. One assumes that this immediate mechanism must, itself, be put in operation by a nervous or chemical stimulus. The stimulus might be a nerve impulse initated at the cerebral or at a lower level. It might be an endocrine product which could activate or inhibit striped or smooth muscle (including the muscle of blood vessels). It might be something which injured the extra-ocular muscles or their nerve supply.

Evidence relating to orbital structures which have been suggested as possible immediate mechanisms of exophthalmos will first be considered. *Smooth muscle mechanisms: 1*) Müller's orbital muscle; *2*) Smooth muscle in the anterior region of the orbit; *3*) Smooth muscle in the posterior region of the orbit; *4*) Tenon's capsule. *Extra-ocular (striated) muscles: 5*) Muscle weakness with a high BMR; *6*) Muscle weakness with a low BMR; *7*) Exophthalmic exophthalmoplegia; *Infiltration mechanisms: 8*) Edema of the orbital tissues; *9*) Fat infiltration. Following this evidence the influence of endocrine products and of the nervous system will be considered and finally reference will be made to lid retraction which has often been mistaken for true exophthalmos.

Smooth Muscle Mechanisms

H. Müller's orbital muscle. An illustration of this muscle as it occurs in man may be found in Whitnall's *Anatomy of the Human Orbit* (134). After carefully defining its limits Whitnall writes: "It is hardly possible that the contraction of such a small muscular mass . . . could affect the position of the eyeball by compression of the mobile orbital fat." Salomonsohn (113) and Hesser (47) had expressed the same opinion. Kraus (60) had mentioned the suggestion that the muscle might have an effect but said that more evidence was needed before any such statement could be made. The small muscle found in man is very different from the strong muscular cone which exists in lower animals. On this account the results of experiments on the dog or cat cannot be applied to man without reservations (18).

Smooth muscle in the anterior part of the orbit. Photomicrographs have been published which show the muscle in the new-born infant (60). They give no support to the suggestion that contraction of the muscle might constrict veins which pass between its fibers towards the spheno-palatine fossa. Kraus mentions the suggestion that the muscle might alter the posterior part of the orbital cavity or the orbital drainage. He adds that it will be the task of experimental and clinical workers to find how far these possibilities are proved to exist. Hesser (47) published photomicrographs of the orbital contents made in many different planes in the orbits of dissecting-room subjects. He declared that Müller's orbital muscle could not produce exophthalmos and that no evidence was known to him which would enable him to decide what was the immediate mechanism.

Cooper (25), in discussing 5 cases of exophthalmos with tachycardia, argued well against the suggestion that the exophthalmos was due to high intraocular pressure. He suggested as cause a morbid excitation of the levator palpebrae superioris which, he said, was "not uncommon in nervous and hysterical females." This increased levator tone has been recognized since Cooper's time and Birch-Hirschfeld (13) reported that an exophthalmos of 0.5 mm. had been produced by voluntarily raising the upper lid. Elevation of the lid has often been mistaken for exophthalmos. It will be considered below.

Landström (65) described his anterior orbital muscle as passing from the orbital septum to the equator of the globe like a ring having its greatest development on the medial side of the orbit. Sattler (115) replied that the function of this muscle was to draw the conjunctiva back during medial and lateral movements of the eye in the horizontal plane, an opinion also held by Whitnall and Beattie (135). Sattler suggested that if Landström's muscle produced exophthalmos it ought to be innervated by the sympathetic nervous system and that, if so innervated, it ought to contract if injections of adrenalin or cocaine were made into it. He made such injections but no exophthalmos followed. The experiments were not entirely conclusive and Landström's views have been accepted by Mulvany (88) who does not offer any new evidence to support them but illustrates his views in a diagram (fig. 3).

Smooth muscle in posterior part of orbit. Hesser (47) published a few photomicrographs from sections made behind the globe. He wrote: "Smooth muscle is found in two positions in the (human) orbit—around the globe and in the inferior orbital fissure. Occasionally definite muscular bundles can, perhaps, be met with also in some other part of the periorbita. I have seen, for instance, an inconsiderable group of a few fibers on the medial wall of the periorbita. But I have never seen smooth muscle on the superior part of the orbit and only in the superior orbital fissure to the same extent as in the inferior orbital fissure." He adds: "I forbear to give a positive opinion on the genesis of this symptom (exophthalmos) since, on the basis of investigations so far completed, it can only be doubtful." D. S. Russell (111) studied serial sections of the whole orbital contents which were situated behind the eyeballs, both in patients who died as the result of accidents and in those of others who died while suffering from Graves' Disease with exophthalmos. She found in all the orbits smooth muscle fibers in the periorbita running roughly parallel to the equator of the eyeball near the postero-lateral wall and the postero-lateral part of

the roof of the orbit. Two of her photomicrographs are reproduced in an article by the writer (18). She found lymphorrhages in the orbits of only one case of fatal Graves' Disease with exophthalmos.

Tenon's capsule. Tenon (127) described a fascial capsule covering the straight and oblique extrinsic muscles of the eye. This capsule was attached in front to the bony margin of the orbit and was reflected back on the globe. Sappey (114), Motais (184), Groyer (43a), and Hesser (47) all claimed to find involuntary muscle fibers in the check ligaments (134). Starling (126) stated that the muscle of Tenon's capsule was innervated by the sympathetic nervous system. Morley (83) has stated his views on the production of exophthalmos in Graves' Disease as follows: "The check ligaments are arranged in continuity with the capsule of Tenon. The check ligaments, as Sappey showed long ago, are chiefly composed of smooth muscle fibers and the capsule, though largely fibrous, can be shown by differential staining to contain a considerable proportion of smooth muscle. These structures, the check ligaments and the capsule of Tenon, combine to form a sort of catapult, which, when it contracts, projects the eyeball forwards." The attraction of this explanation depends on a belief that the anterior points of attachment of the check ligaments can really form fixation points for the catapult elastic. Whitnall's (134) description of the attachments and his illustrations on pages 129, 147, 289, and 294, should be compared carefully with the illustrations in Duke-Elder (30) on pages 178–179 and with figure 2 of Mulvany's (88) article on page 599. The position of the equator of the globe relative to the anterior orbital margin may well decide the value of this hypothesis

It is tempting to imagine that smooth muscle fibers somewhere in the orbit might be made to contract through stimulation of the cervical sympathetic nerve trunk, but stimulation of this trunk fails to produce exophthalmus in human subjects or higher apes, though an analogous procedure will produce exophthalmos in dogs and cats (99).

WEAKNESS OF EXTRA-OCULAR MUSCLES.

Plummer and Wilder (97) in a noteworthy review distinguished exophthalmos with a high BMR from exophthalmos with a low BMR. This distinction had been revealed by a number of reported cases where 'thyrotoxic' exophthalmos had persisted or increased after thyroidectomy (20, 29, 91, 107, 139). Evidence from animal experiments which has been reviewed above already suggested a connection between exophthalmos with a low BMR and the anterior pituitary body. Other conditions in which exophthalmos may exist with a low BMR include myxedema, exophthalmic ophthalmoplegia, bulbar paralysis and myasthenia.

Exophthalmos and muscular weakness with high BMR. Cooper (25) stated that Dalrymple believed that the exophthalmos of Graves' Disease was due to weakness of the extrinsic muscles of the eye. Möbius (80) agreed. Askarazy (2) examined various skeletal muscles from 4 patients who died of Graves' Disease and reported that degenerative changes might be found in any striated muscle except that of the heart. The unbelievers suspected post-mortem changes. When successful thyroidectomies became common, the orbital contents available for examination post-mortem became fewer. L. B. Wilson in a personal communication to Plummer and

Wilder (97) stated that in post-mortem examinations made on 8 patients who died of active Graves' Disease before 1917 he had found little orbital fat or edema but had found small and degenerated muscles in the orbital cavity and also weak muscles in the thigh. Plummer and Wilder's table 7 gave figures relating the degree of exophthalmos to a physiological test for thigh muscle weakness in 1000 cases seen before 1922 and 1000 cases seen after 1930. The authors reported a positive correlation between weakness of the quadriceps extensor muscles and exophthalmos. Weakness of extra-ocular muscles has also been noted by Brain (16) and Mulvany (87, 88). Infiltration of fat has also been found in cases of thyrotoxicosis (109) and will be considered under fat infiltration below. The infiltration of water or fat after thyroidectomy will be considered in relation to anterior pituitary thyrotrophin.

Exophthalmos and muscular weakness with low BMR. Exophthalmos appears with a low BMR in the following cases: *1*) primary thyrotoxicosis followed by thyroidectomy; *2*) primary thyrotoxicosis followed by atrophy of the thyroid gland; *3*) those which seem explicable by primary thyroid deficiency and relative overaction of the anterior pituitary; and *4*) those which seem explicable by simultaneous thyrotoxicosis and over-production of APTH (72). The essential feature of all these cases is an active anterior pituitary gland which alters the condition of the orbital muscles and connective tissues in much the same way as it has been shown to do in thyroidectomized guinea-pigs (120). The condition of the muscle in post-operative cases will be described here. The evidence which connects the condition with the pituitary gland will be given later.

Burch (20) reported a case of a male aged 62 who came with 7 mm. exophthalmos 2 years after a sub-total thyroidectomy and with right eye exenterated. The extra-ocular muscles were greatly enlarged and exhibited fiber degeneration, edema and lymphocytic infiltration. Degenerated fibers had been replaced by fibrous tissue. The orbital fat, connective tissue and nerves seemed normal. Burch referred to previous authors who had found some of these conditions in goitre cases. Naffziger (90) examined and operated on the orbits of 6 patients who suffered from severe exophthalmos after removal of their thyroid glands. The orbits showed edema of both the cellulo-adipose tissue and the muscles with some infiltration of small round cells. There was also some edema of the lids and conjunctiva with lacrimation and impairment of the movements of the eyeball. Swelling of the optic disc was present in 5 of the cases. Orbital decompression was performed through the orbital roof. Some connective tissue and fat were removed from the posterior part of the orbit as well as portions of extrinsic muscles. The muscles were swollen so as to reach 3 to 8 times their normal size. Microscopical examination of them revealed edema, loss of striation, collagen in interfibrillar spaces and collections of small round cells the size of lymphocytes near the blood vessels. The operation reduced the exophthalmos by amounts which varied from 2 to 7 degrees, presumably 2 to 7 mm., on the exophthalmometer. It relieved the edema of the orbit and the aching sensation which 3 of the patients had felt and had localized behind the eyes. The exophthalmos did not recur. In the discussion on Naffziger's paper Katz (90) described a similar case in which operation had produced 5 mm. reduction in exophthalmos.

Other cases of post-thyroidectomy exophthalmos have been described (37, 45, 87, 88, 92, 107, 108, 139, 140). Thomas and Woods (128) reported on 18 such cases and Marine (73) collected 56 such cases of which 60 per cent were in males. Muscle changes were prominent in nearly all of these cases.

Exophthalmic ophthalmoplegia. Under the name of exophthalmic ophthalmoplegia, Brain (16) grouped 31 patients who showed exophthalmos and ophthalmoplegia. In 29 of these cases the exophthalmos was the first cause of complaint; in two cases the exophthalmos began after thyroidectomy. Brain believed that in these cases thyrotoxicosis played no essential part since thyrotoxic symptoms were generally slight or absent and the BMR was normal or subnormal. Treatment by thyroidectomy was ineffective. In distinction to myasthenia gravis the paralysis was usually limited to the ocular muscles. The muscle weakness was not characteristic of myasthenia and was not relieved by administration of physostigmin. The ophthalmoplegia was attributed to the mechanical effect of the intra-orbital tension on the muscles. Brain stated that *1*) eye symptoms are usually the first cause of complaint; *2*) the average reading of the exophthalmometer tends to be higher than in hyperthyroidism (7 mm. compared to 2 mm.), but the patient's placid appearance is in striking contrast to the anxious expression of the typical patient with exophthalmic goiter; *3*) in 13 out of 18 cases the ophthalmoplegia was unilateral and in all these 13 the elevating muscles were weak or paralyzed whereas abduction was only reduced in 4 cases and adduction or depression in one case each; *4*) in no case at the time of study was there evidence of any lesion of the central nervous system though in one case there had been generalized muscular wasting which had recovered completely after partial thyroidectomy; *5*) cutaneous pigmentation was often noted.

The extra-ocular muscles were only available for examination in two of Brain's cases. In one case (*case 1*) their appearance suggested edema and there were areas of infiltration by leucocytes. The patient's thyroid gland showed the changes characteristic of Graves' Disease. Death had been due to pneumococcal pericarditis. The second case (*case 6*) had previously undergone thyroidectomy. His thyroid when examined after operation suggested thyrotoxicosis. When he was being treated for exophthalmic ophthalmoplegia a fragment of his levator palpebrae muscle was removed at biopsy. Compared with a control muscle prepared similarly for microscopic examination its fibers showed changes in cross striation and staining. The mode of the diameters of 100 fibers was 25 μ as compared to a mode of 15 μ in the control muscle.

Unfortunately no details were given to show the distribution of signs in the 31 individual cases, but, apart from the two which were post-thyroidectomy cases, the other 29 might have been divided into those with and without signs of thyrotoxicosis such as rapid pulse, moist skin, tremor of hands and high BMR. If this had been done the cases of exophthalmic ophthalmoplegia might all have been classified as either thyrotoxic exophthalmos with raised BMR, post-thyroidectomy exophthalmos, or primary thyrotoxicosis followed by functional decadence of the thyroid gland. The ophthalmoplegia might have been due either to thyrotoxicosis or to the changes in the muscles which occur when APTH is not neutralized by thyroxin.

Brain's statements, often modified, have been put inside quotation marks by

Mulvany (88) and have been vigorously criticized. Those who are interested should read the papers for themselves. Mulvany considered that "the term exophthalmic ophthalmoplegia . . . could appropriately be dropped for the more accurate designation of its two components; thyrotoxic ophthalmoplegia and thyrotrophic ophthalmoplegia" (88). The cases which he gives as examples of these two types of ophthalmoplegia might serve as examples of Plummer and Wilder's classification of exophthalmos with high or low BMR.

Infiltration Mechanisms

Edema of the orbital tissues. The title given to Basedow's (9) classical article of 1840 was "Exophthalmos Due to Hypertrophy of the Connective Tissue of the Orbit". The following passages are translated from it: "I have already given my opinion on the immediate cause of the exophthalmos that it is a strumous hypertrophy of the cellular tissue behind the globe, in the words of St. Yves 'un amas d'humeurs qui se font derrière la globe de l'oeil'. Yet I hold that this hypertrophy is a secondary sign of a diseased circulation and of a faulty crasis (*sic*) of the blood, a dyscrasia which, by means of the scrophule already described, appears in diseased vegetative glands and their tissue". In Basedow's time the coagulating property of the blood was considered to be of importance for general health and references to this may be found in contemporary English medical literature. Microscopical examinations were not possible in Basedow's cases and Basedow does not pretend to know which cell constituents of the connective tissue, if any, hypertrophy.

In lower animals exophthalmos has been produced experimentally by producing an imbalance between the blood supply to the orbital cavity and the drainage of the cavity. This imbalance may be due to a rise of arterial blood pressure (19), to interference with the venous outflow (69) or to dilatation of the orbital arteries by acetyl choline (19). High arterial blood pressure is not noticeable in Graves' Disease and patients suffering from high blood pressure do not usually exhibit exophthalmos so it is not likely to be responsible for the exophthalmos. By obstruction of the anterior orbital vein when the subject's head was upright, Birch-Hirshfeld (13) produced 1 mm. of proptosis. Obstruction of orbital veins has never been shown in Graves' Disease but its occurrence has not been disproved. Basedow (9) and Trousseau (129) certainly believed that the blood supply to the orbit was increased in Graves' Disease. Fuchs (38) suggested dilatation of the carotid arterial tree. Arterial dilatation seems at first thought improbable in a state which includes some stimulation of the sympathetic nervous system (the nerve to the superior tarsal muscle) but circulatory imbalance may possibly be a factor in the exophthalmos of Graves' Disease. It almost certainly occurs in post-thyroidectomy exophthalmos as a result of muscle hypertrophy and venous obstruction.

Fat infiltration. The orbits examined by Naffziger came from cases of exophthalmos which recurred or increased after thyroidectomy. The chief changes seen in them were increase in the size of the extra-ocular muscles, lymphocytes and the edema which has been mentioned. Smelser (120) obtained very similar results of guinea-pigs which were treated with APTH after thyroidectomy but he mentions solid masses of fat fairly free from ordinary connective tissue. Paulson (95) confirms this

work on guinea-pigs. Deposition of fat after thyroidectomy seemed possible but it was with surprise that the writer learned that deposition of fat also occurs in thyrotoxic cases. Rundle and Pochin (109) described 17 cases where the patient died while suffering from Graves' Disease with or without some exophthalmos. The extrinsic muscles, lacrimal glands and residual tissues were weighed separately, dried at 108°F. to constant weight and then extracted with chloroform or ether in a soxhlet apparatus. The water content expressed as a percentage of the fat-free weight of the structure was practically identical in the muscles and lachrymal glands of normal and thyrotoxic subjects. It was slightly *lower* in the 'residual tissue' of the thyrotoxic cases than in those of the normal cases. In this respect the results were different from those of Smelser (120) when APTH was given to thyroidectomized animals. Rundle and Pochin report that "exophthalmos in Graves' Disease is accounted for quantitatively by an increase in bulk of the retro-bulbar tissues. The increase is relatively greatest in the eye muscles of which the average fat content was doubled in a series of 17 thyrotoxic cases. . . . Increase of fat in the orbital fatty tissue is, however, responsible for *most* of the increase in bulk". Control orbits from obese patients showed no increase of orbital fat. The authors are to be congratulated on obtaining for study so many suitable orbits. Rundle and Wilson (110) studied further the bulging of eyelids with exophthalmos and showed that prolapsing orbital fat as well as edema could cause the sign.

Increased fat content had previously been offered as an explanation of exophthalmos in Graves' Disease by Askarazy (2), Bristowe (17), Mendel (78), Murray (89) and Mackinnon (70). Each of the last two authors described a single case of Graves' Disease in which exophthalmos was accompanied by increased orbital fat. In Murray's case one of the orbits contained half an ounce (14 gm.) of fat. Neither of these cases was seen at the initial stage of exophthalmos. Cases of Graves' Disease have also been examined in which no abnormal fat could be found (51, 93, 111). This fact makes it difficult to accept Smelser's suggestion (120) that both hyper- and hypothyroid exophthalmos are produced in the same way.

ENDOCRINE PRODUCTS AND EXOPHTHALMOS

For the study of endocrine extracts the dog, cat, guinea-pig and rabbit were used before human subjects. Owing to anatomical differences between human and sub-human orbits, results based on the latter may not always apply to man (18). Further, in observing patients investigators did not usually distinguish between true exophthalmos and lid retraction (Dalrymple's sign) in Graves' Disease. Lid retraction often gives the impression of exophthalmos when under observation or in full-face photographs. This has been recognized by many thoughtful observers of Mr. Eddie Cantor and of the British actors Mr. George Robey and Mr. Leslie Henson. Fewer people have recognized that even photographs taken from the side (at right angles to the face) can suggest exophthalmos when only lid retraction is present. The method of Birch-Hirshfeld (13) cannot be used for hospital cases as it demands the accurate fitting of a lined metal helmet to each subject's head and a rigid fitting of the camera to the helmet. Exophthalmometers have been described by Hertel (46) and Pochin (99). With these a series of readings can be made from a

base line of the lower orbital rim or the outer orbital rim. Plummer and Wilder (97) noted cases in which "one eye seems to be more prominent than the other, but the exophthalmometer readings are identical and the apparent prominence is entirely due to the retraction of the lids". Measurement of the lid retraction has been studied by Pochin (98, 100) and will be considered later.

Thyroid extracts. The earliest experiments with thyroid extracts were made by patients who took them in order to reduce their weight. Moorhead (82) described the case of a woman who had taken thyroid extract for over two years and came to an oculist suffering from exophthalmos and thyrotoxicosis. Justin-Besançon and others (53) quote similar cases of Béclère and Rist. Brain (15) described the case of a woman who produced unilateral exophthalmos by taking thyroid extract over a long period and whose condition was cured by ceasing to take it. In Brain's case the exophthalmos was genuine and not merely lid retraction. Brain could only find in the literature about 20 cases where the exophthalmos was stated to be due to thyroid extract and some writers have denied that thyroid extract can produce exophthalmos. They have been upheld in their denial by cases of Graves' Disease in which no exophthalmos is present and other cases where thyroidectomy has failed to relieve the exophthalmos though the heart and metabolic rates fell after the operation. Seitchik (116) mentions 11 cases of exophthalmos which followed the administration of thyroid extract, taken in 4 cases for the relief of obesity and in 7 cases for the relief of myxedema. Plummer and Wilder (97) published figures to show that the frequency of exophthalmos in 1000 cases before 1922 and in another series of 1000 cases seen after 1930 varied with the BMR and so, presumably, with the activity of the thyroid gland. They agree that quite exceptional cases occur. *Case 5* described by Brain (16) had been treated by partial thyroidectomy for chronic thyrotoxic myopathy. "The body musculature recovered completely after a partial thyroidectomy", he stated. The increase of fat which Rundle and Pochin (109) measured in the orbits of patients who had died while suffering from Graves' Disease is further evidence of a connection between thyroxin and increased fat content of the orbit. This increase of fat has not been reported in cases of post-thyroidectomy exophthalmos, so far as the writer knows.

Thyroxin and adrenalin. An article by Sainton and Hesse (112) describes the production of exophthalmos and other symptoms of Graves' Disease in a myxedematous woman aged 38 by a rather strenuous treatment with thyroxin and adrenalin. The article is illustrated by full-face photographs only, but as pseudo-exophthalmos was recognized by workers in Paris at that date, one may assume that the exophthalmos was a true one. Justin-Besançon and others (53) describe the temporary appearance of the exophthalmos in a patient with Graves' Disease but normal eyes when ephedrine was administered. (Ephedrine delays the inactivation of adrenalin (39) as physostigmin delays the inactivation of acetyl choline.) Urechia and Retezeanu (130) reported the temporary production of moderate exophthalmos when thyroid and ephetonine were given at the same time to a patient.

One might distinguish the causation of these types of exophthalmos by naming them 'thyrotoxic exophthalmos' (88) in the patients who took thyroid extract only, 'thyro-adrenalin exophthalmos' in Sainton and Hesse's case and 'thyro-ephedrine

exophthalmos' in the cases of Justin-Besançon and Urechia and Retezeanu. Brain's case (15) might perhaps be included as a case of thyro-adrenal exophthalmos since facial hair suggested hyperactivity of the adrenal cortex.

Anterior pituitary gland extracts. Anterior pituitary gland extracts were first used to produce exophthalmos in rabbits and guinea-pigs (35, 68, 75, 104). Smelser (120) published photomicrographs to illustrate the similarity between orbital tissues in thyroidectomized guinea-pigs so treated and the orbits of patients who had exophthalmos after thyroidectomies. Both in guinea-pigs and in human subjects there was edema in the connective tissues and between the muscle fibers. More degeneration of muscle fibers was present in the guinea-pig than in the human orbits.

Neither Smelser nor Pochin (101) who also used guinea-pigs reported any increase of orbital fat as the result of APTH. In human subjects Mulvany (87, 88) contrasted 'thyrotoxic' and 'thyrotrophic' exophthalmos, associated respectively with thyrotoxicosis and excess of APTH. In the latter condition "the orbital fat is little disturbed except in the presence of a generalized edema when it may become infiltrated with round cells". In the former condition the orbital fat is "normal or slightly increased". Mulvany prepared a table of other differences in the two types of exophthalmos. Beside his suggested name of 'thyrotrophic exophthalmos' the condition has been called 'thyrotopic exophthalmos' by Zondek and Ticho (141), 'paradoxical exophthalmos', 'pituitary exophthalmos' (141) and 'low metabolism exophthalmos' (97). Two pieces of evidence seem to support directly the relation of the exophthalmos to the pituitary gland. Mandeville (71) has reported the relief of post-thyroidectomy exophthalmos by irradiation of the hypophysis. The eyeballs receded 5 mm. He quotes good results reported by Ruedemann (107) and by Thomas and Woods (128) but disappointing results reported by Merrill and Oaks (79) and by Naffziger (90). A crucial experiment was described by Stallard (125). A male patient aged 31 suffered from exophthalmic ophthalmoplegia with a low basal metabolic rate. He was given APTH as a diagnostic test. His BMR rose from about normal to about plus 40 per cent; he showed the signs of Graves' Disease; the exophthalmos increased; a corneal ulcer which resisted treatment perforated and it was necessary to remove the eyeball. The extra-ocular muscles were from 4 to 5 times their normal size. Part of a muscle was removed and showed chronic inflammation with small round cells and interstitial fibrosis but no definite degeneration of the muscle fibers. The action of APTH would seem clear in this case but one point remains unexplained: if it is true that an increased amount of APTH circulates in the blood in myxedema (141), why do not patients with myxedema suffer from exophthalmos? Is it due to lack of adrenal products?

Mann (72) has mentioned cases beside those of post-thyroidectomy exophthalmos which seem to be related to the pituitary body. In 4 cases the symptoms suggested primary thyrotoxicosis followed by atrophy of the thyroid gland. In another 4 cases there was primary thyroid deficiency with excess APTH. All these 4 patients had sallow, thick coarse skin suggesting myxedema. In three of the cases the lids would not close completely but this was due to their swelling and stiffness rather than to retraction. The BMR was measured in three cases and was found to be −9 per cent, −27 per cent and about −1 per cent respectively. Three cases showed proptosis.

Treatment by tarsorraphy, excision of conjunctiva and thyroid extract saved the remaining eyes. (Stallard's case (125) may have been an early case of primary thyroid deficiency.) Finally Mann mentions four cases showing loss of weight, tachycardia, proptosis and ophthalmoplegia co-existent. In three cases the BMR's were respectively +38 per cent, +42 per cent and +43 per cent; in the fourth case the BMR was not measured before treatment. These were thought to be due to a combination of thyrotoxicosis and excess of APTH.

Gonadal products. Certain gonadal products have been shown to increase exophthalmos in animal experiments but up till now the writer has seen no reports of their action in cases of human exophthalmos.

MOTONEURONES IN GRAVES' DISEASE WITH EXOPHTHALMOS

The appearance of exophthalmos both as part of Graves' Disease and after thyroidectomy has often been related in time to emotional shock. (Sollwig quoted Edmunds, 32; Geyer quoted Voss, 131.) In most of the cases which have been reported careful distinction was not made between true exophthalmos and retraction of the upper eyelid but in some cases the exophthalmos was certainly genuine.

It has been natural to seek to explain exophthalmos by the sympathetic nervous system which supplies the smooth part of the levator palpebrae superioris (the superior tarsal muscle). Yet as we have said above stimulation of the cervical sympathetic nerve trunk does not produce exophthalmos in man. The possibility of paralysis following injury to the nerve supply of the extrinsic muscles of the eye was mentioned by Ballet (5) who quoted the opinion of Sattler (115) and others that the exophthalmos of Graves' Disease was due to 'functional disease of the bulb'. Greenfield (43) reported changes in the cervical sympathetic ganglia. L. B. Wilson (136) found hyperpigmentation, granular degeneration, atrophy and reduction of ganglion cells varying with the duration and remissions of the disease in the ganglia of 12 patients who died of Graves' Disease. He produced similar cell pictures by innoculation of bacteria into or frequent stimulation of the superior cervical ganglion (137). Later he referred to examinations made before 1917 on the eye muscles and expressed the tentative opinion that the exophthalmos could be explained by weakness of the recti muscles (138). Heuer (48) thought that the eye-muscle palsies in cases of exophthalmic goiter might be due to cerebral disturbances but had no material for examination. In one such case Wedd and Permar (132) made serial sections from 25 areas between the basal ganglia and the medulla without finding anything abnormal. Risak (105) reported a case where all the symptoms of Basedow's Disease followed an attack of encephalitis lethargica. Penfield (96) reported a case in which exophthalmos and other symptoms followed a diencephalic tumor close to the area of the hypothalamus delimited by Bard. During the attacks the eyeballs were said to protrude. This protrusion, if actual, may have been due to increased intracranial pressure, to nerve injuries which removed controlling impulses from lower nerve paths or to the removal of inhibitions from the pituitary body and, in consequence, an increased production of APTH.

Apart from possible nerve injuries Brain (16) refers to thyrotoxic myopathies: acute, chronic and periodic as well as to myasthenia gravis. Brain's *case 5* appears

to have been a case of general thyrotoxic myopathy followed by post-thyroidectomy exophthalmos. The myopathies are distinguished from exophthalmic ophthalmoplegia by their cure if thyroidectomy is possible. Ophthalmoplegia with Graves' Disease may respond to treatment with physostigmin (in which case Brain considers it to be due to myasthenia) or may fail to do so either because it has a different cause or, in some cases, because the treatment is given too late. Cases of myasthenia with exophthalmos have been recorded by L. B. Cox (26), by H. Zondek (140) and by Laurent (66). Rundle and Pochin (109) say that "it appears probable that lid retraction, ophthalmoplegia and possibly other eye signs may prove to be related in some way to the extensive increase in fat content of the extrinsic muscles in thyrotoxicosis".

LID RETRACTION

Before summing up the work which has been done on exophthalmos, reference must be made to studies on lid retraction. Both upper and lower lids possess smooth retractor muscles which were described as Müller's palpebral muscles to distinguish them from his orbital muscle. Müller's palpebral muscle of the upper lid is also named the smooth part of the levator palpebrae superioris or the superior tarsal muscle. Pochin (100) describes its origin from the striated fibers of the Levator and its passage forwards to be inserted into the upper border of the tarsal plate. This plate in turn is attached to the skin of the lid at its free lower border, the anterior surface of the tarsus being unattached to the super-jacent skin. Into this part of the skin is inserted the tendon of the striated Levator palpebrae. The superior tarsal muscle is innervated through the cervical sympathetic nerve trunk. In Graves' Disease lid retraction due to it alone may occur without exophthalmos, or exophthalmos may occur without lid retraction (98, 100). At death lid spasm disappears but exophthalmos does not (109). Sympathectomy improves lid retraction but not exophthalmos (117). After sympathectomy voluntary lid retraction can still be performed (100). In Horner's (50) Syndrome as originally described enophthalmos was unimportant but ptosis was definite (99). Electrical stimulation of the cervical sympathetic trunk in human subjects produces lid retraction but not exophthalmos (99). Eden and Trotter (31) noted the presence of lid retraction in 52 per cent of 134 cases of toxic diffuse goiter. The appearance of exophthalmos so produced has already been emphasized. Leriche and Fontaine (67) believed that lid retraction might be stimulated not only by fibers in the cervical sympathetic trunk but also by others which were believed to travel along the course of the vertebral artery. They also described interesting symptoms produced by a neuroma of the cervical sympathetic nerve trunk after removal of the superior cervical ganglion. Mulvany (88) included lid retraction among the signs of thyrotoxicosis and stated that it was absent in uncomplicated thyrotropic exophthalmos. Rundle and Pochin (109) drew attention to the fact that in Graves' Disease the fatty deposits were relatively greater in the levator palpebrae superioris muscle than in other extra-ocular muscles and suggest that 'lid retraction, ophthalmoplegia and possibly other eye signs may prove to be related to the extensive increase in fat content of the extrinsic muscles in thyrotoxicosis.' The same authors have drawn attention to the fact

that bulging of the eyelids in Graves' Disease may be due to overfilling of the orbital cavity so that the orbital fat comes forward as it does also when tumors are present in the orbit. They distinguish this fatty swelling from edema of the lids (110).

SUMMARY

In lower animals exophthalmos may be produced experimentally by stimulation of the cervical sympathetic nerve trunk, by administration of thyroxin together with certain sympathomimetic substances or by the use of cyanides. These act by causing contraction of Müller's orbital muscle which, in turn, increases the fluid tension behind the eyeball and drives it forward. Almost the whole of the nervous path is known from the hypothalamic region to Müller's orbital muscle. In apes, stimulation of the cervical sympathetic trunk fails to produce exophthalmos. Exophthalmos may also be produced in the absence of the thyroid gland by anterior pituitary thyrotropic hormone (APTH). It is associated with edema of the orbital contents. The effect of APTH is increased by gonadal substances.

True exophthalmos in Graves' Disease must be distinguished from lid retraction by means of an exophthalmometer. It may exist with a high or with a low BMR. Evidence shows that the form with a high BMR is accompanied by considerable increase of orbital fatty tissue and increase of fat in the extra-ocular muscles. Evidence suggests that this form may be produced by the administration of thyroxin (82, 15). It is usually but not always accompanied by lid retraction. It has been produced in a hypothyroid patient by administration of thyroxin and adrenalin (112) as well as by thyroxin and ephedrine or ephetonine (54, 130). Other possible but unproven factors in its causation are smooth muscle in the orbit or disturbances of the orbital circulation. Müller's orbital muscle is not responsible for exophthalmos in Graves' Disease.

Exophthalmos with a low BMR (hypothyroid, post-thyroidectomy, thyrotropic exophthalmos) is due to the action of an anterior pituitary substance (APTH) which produces edema and round cell deposits in the loose orbital tissue and in the extrinsic muscles, sometimes increasing their volume to such an extent that not only is the eyeball pushed forward but the circulation in the orbit is obstructed. It is uncertain whether this muscle change is primary or follows changes in the orbital connective tissues as it did in Smelser's guinea pigs.

Brief consideration has been given to the occurrence of exophthalmos due to ophthalmoplegia and myasthenia. Treatment of the condition has not been considered.

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